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Detection of Circulating Immune Complexes (CIC); With Results From Selected Disorders by Raji Cell Radioimmunoassay (RIA)

by

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A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Detection of Circulating Immune Complexes (CIC); With Results From Selected Clinical Disorders by Raji Cell Radioimmunoassay (RIA)", submitted by Mrinal K. Dasgupta, in partial fulfilment of the requirements for the degree of Master of Science in Experimental Medicine.

To my wife

Shibani

and

our children

Bonnie and Tina

Abstract

Raji cell radioimmunoassay (Raji-RIA), a sensitive technique for the detection of circulating immune complexes (CIC), has been developed and modified in terms of standardization without the use of aggregated human IgG (AHG). Raji-RIA results were then tested for their reproducibility and compared with results of two other CIC assays [^{125}I-C1q binding activity (C1q-BA) and ^{125}I bovine conglutinin binding assay] in normal and pathological sera.

In evaluating the false positivity by antilymphocytic antibodies in Raji-RIA, antibody dependent cellular cytotoxicity (ADCC) assay with ^{51}Cr-labelled Raji cell targets (ADCC-Raji) was used. Results indicate minimal influence of such antibodies under the test conditions of Raji-RIA in SLE, renal transplant and multiparous womens' sera.

Raji-RIA was applied in conjunction with C1q-BA in three different clinical situations and the following observations were made:

a) prevalence of CIC in each group of disorders were significantly higher than respective control groups with Raji-RIA showing more positive results;
b) in cystic fibrosis patients, a subgroup could be identified having immune complex-mediated lung injury, based on the higher prevalence of CIC levels;
c) in hemodialysis, patients' sera were found <u>not to</u> contain DNA antibodies in free or complex form, clarifying a misconception in the literature; and
d) in MS patients, prevalence of CIC was found to be significantly increased in active states of the disease compared with non-MS neurologic controls.

Preliminary attempts were made to characterize myelin basic protein (MBP) as an antigenic constituent of the CIC eluted from the Raji cells. MBP containing complexes were noted in MS more frequently than in controls, in accordance with the proposed autoimmune nature of the disease.

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LIST OF ABBREVIATIONS USED

AHG - aggregated human IgG

ADCC - antibody dependent cellular cytotoxicity

BA - binding activity

BSA - bovine serum albumin

CDC - complement dependent cytotoxicity

CF - cystic fibrosis

CIC - circulating immune complex

Clq-BA - ^{125}I Clq binding activity (fluid phase assay)

HD - hemodialysis

IC - immune complex

MBP - myelin basic protein

MS - multiple sclerosis

NHS - normal human serum

PA - pseudomonas aueroginosa

PEG - polyethylene glycol

PF - pulmonary function

polydAT - polydeoxyadenylate-deoxythymidylate

PAGE - polyacrylamide gel electrophoresis

RA - rheumatoid arthritis

RIA - radioimmunoassay

RID - radioimmunodiffusion

SDS - sodium dodecyl sulfate

SLE - systemic lupus erythematosus

SP - solid phase

Chapter I: Introduction and Review of Literature

A. Introduction

Immune complex (IC) formation in vivo occurs from interaction of various endogenous or exogenous antigens with their corresponding antibodies in human beings. Formation of such complexes in normal persons, at a certain level, probably represents a physiologic immune mechanism designed to eliminate or neutralize antigens and thus protects the host.

Von Pirquet in 1911 (209) first suggested the harmful role of ICs in serum sickness. Later, experimental demonstration of IC formation with vasculitis and glomerulonephritis in serum sickness was provided by Germuth (55) and Dixon (31) in the 1950s. Since then a considerable amount of evidence of IC-mediated injury has been described in various autoimmune, infectious and malignant disorders, principally by way of immunohistopathologic demonstrations of ICs in target tissues.

Recent developments in the detection of ICs in serum (circulating immune complexes: CIC) and other biological fluids have created an area of considerable scientific interest to the clinician as well as the basic immunologist. Various physicochemical and biologic methods of CIC detection have been described, varying in their sensitivity and principles of detection. The biologic methods are more sensitive and have specificity in terms of Ig class or complement component in the IC. They are not specific for the antigen(s) in the IC. Only a few are suitable for routine use because of their sensitivity and reproducibility (WHO study, 1978; 97).

A vast amount of information has been accumulated in the last several years demonstrating the prevalence of CICs in various clinical conditions such as autoimmunity, infections, malignancies, etc., where

presumably persistent antigenemia and optimal host immune response give rise to the formation of such complexes (Appendix 1).

One of the aims of CIC research is to determine its usefulness for monitoring disease activity in CIC-mediated disorders. So far this has been shown to be helpful in disorders such as systemic lupus erythematosus [SLE (3, 5, 23, 35, 68)], rheumatoid arthritis (143), lyme arthritis (67), infective endocarditis (13, 14, 122), and certain types of leukemias and lymphomas (24, 161). Another objective is to isolate and characterize in vivo formed complexes to determine their specific role in the pathogenesis of these disorders. Limited progress has been made in this area but, with increasing technical improvements, methods of isolation of such complexes have been achieved [eg. elution of ICs absorbed onto Raji cells (196), or isolation from ligand bound complexes (26), etc.]. With perfection of these techniques, the ultimate goal of antigen purification for animal immunization and vaccine production may become possible.

In this thesis, the application of CIC techniques in three clinical situations [multiple sclerosis (MS), hemodialysis, and cystic fibrosis (CS)] will be evaluated with the Raji cell radioimmunoassay [Raji-RIA, (199)], a highly sensitive and reproducible technique. Since a single CIC assay is not dependable for detecting all types of complexes, another sensitive technique, C1q binding activity [C1q-BA (231)], based on differing biologic principles, was also included. Results indicate that application of CIC study in these three clinical situations are useful and, more importantly, the use of Raji cells to isolate antigen from CICs appears promising.

B. Review of the Literature

1. Methods for CIC Detection

Binding of antigen to antibody occurs forming non-covalent bonds and leads to formation of altered molecular and functional characteristics of the combined molecules (IC) by (i) increase in molecular size, changes in surface properties, charges and solubility; (ii) activation of rheumatoid factor (RF), complement system; (iii) activation of other biologic systems, eg. kinins, enzymes, etc., and; (iv) binding to cellular or tissue receptors for Ig-Fc, C_{3b}, C_{3d}, C_{5a}, etc., and thus modulates immunoregulation by its effect on the lymphoid system influencing ADCC activity, suppressor T-cell function, etc. (194).

Laboratory methods to detect IC are based on these above-mentioned properties of the complexes and are generally divided into four categories:

a. Physical and physicochemical

i) Ultracentrifugation - IC are separated in fractions larger than free Ig (179);

ii) Gel filtration - IC are separated in fractions larger than free Ig (95);

iii) Selective precipitation of complexes from free Ig or other macromolecules, eg. ammonium sulfate precipitation (47), polyethylene glycol precipitation (230) and cryoprecipitation (223).

All these methods lack immunologic specificity and are therefore unsuitable for routine use alone, but may be used as the preparative stage for other methods or combined with other biologic methods to ensure immunologic specificity: viz

i) Raji or Clq-BA in ultracentrifuged fractions of serum (146, 199),

ii) Ultracentrifugation followed by RID for Ig (21),

iii) PEG precipitation followed by RID for Ig (40, 84), C_3 C_4 (40).

b. <u>Tests that are based on the interaction of ligands in vitro to in vivo formed complexes</u>

i) Rheumatoid factor binding assay (6b, 108, 220),

ii) Clq (human) binding assay (6a, 69, 142, 231),

iii) Bovine conglutinin binding assay (25, 45, 85, 109),

iv) Staph. Protein A binding assay (65, 118),

v) C_3 binding solid phase assay (155).

These methods are based on immunological reactions and are used routinely. Detailed characteristics of some of these are given in Table 1.

c. <u>Binding to biologic receptors</u>

IC's with or without fixed complement will bind in vitro to cellular receptors and are then detected by radiolabelled or enzyme labelled antibodies to human Ig. They are very sensitive but require live cells. Antibody to cell membrane antigens present in the test sera is a drawback of these methods as listed below:

i) Raji cell RIA (199),

ii) Macrophage inhibition assay (128),

iii) Human red cell assay (204),

iv) Polymorphonuclear binding test (91),

v) Other cell line derived tests (157).

METHODS & REFERENCES	PRINCIPLES	DETECTION & QUANTITATION	ANTIBODY CLASS			SIZE		†COMPLEMENT	DISADVANTAGES & FALSE POSITIVES	ADVANTAGES
			IgG	IgM	IgA	>19S<				
1. ULTRACENTRIFUGATION (UC) (179)	Molecular weight	UC ≥ 20,000 g				+	+	-	No immunologic specificity Not suitable for routine tests	Preparative
2. ULTRACENTRIFUGATION + radial immunodiffusion (RID) (21)	Molecular weight, in acid and normal pH	" + RID with specific anti -Ig	+	+	+	+	+	-	Not suitable for routine use	● Not influenced by Ig aggregates
3. POLYETHYLENE GLYCOL a) PEG precipitation (30, 40) b) + RID (40, 84)	? ? Cryoprecipitation ? ? Electrical charge	a) OD 280 nm b) RID with anti Ig	+			+ +		-	Aggregation of serum Ig Lipoproteins Serum levels of Ig	Simple, ideal for screening ●
4. ADCC INHIBITION (87)	Inhibition of effector cell activity via Fcr by IC	Cr release from target cells	+			+	+	-	Heat inactivation of sera, allo or autoantibodies Selected donor needed	Sensitive Fcr binding test
5. Clq BINDING ACTIVITY (BA) a) Solid phase assay (69, 205) b) Fluid phase assay (142, 231)	IC binding to Clq coated plastic tubes ^{125}I Clq binds to Ig of IC	^{125}I anti-IgG Pptn. by 2-3% PEG	+ +	 +		+ +		+ +	a) Detects IgG complexes b) Endotoxins, Polyanions, Heparin, C-Reactive proteins, Fibrinogens + PEG effects	a) Highly sensitive b) IgG + IgM IC Simple Rapid
6. BOVINE CONGLUTININ BA a) Solid phase (24, 45) b) Fluid phase (85, 109)	a) C3bi fixed IC binds to Conglutinin coated plastics b) ^{125}I-Conglutinin binds to C3bi of IC	^{125}I anti -IgG or Enzyme labelled Ig Pptn. 2-3% PEG	+ +	 +				+ +	a) High background, influenced by proteases	● a) Highly sensitive Use of non-human ligand. b) IgG+IgM IC Simple, could be combined with other PEG tests.
7. RAJI CELL* RADIO IMMUNO ASSAY (RIA) (199)	C3 fixed IC binds via avid C3b and C3d receptors	^{125}I- anti IgG	+			+	+	+	Cell culture Anti lymphocytic antibodies	● Highly sensitive

* Raji cells: Human lymphoblastoid cell line derived, has avid C3b, C3d receptors besides less avid Clq, C5a and IgG Fcr. No surface Ig.
† Complement: complement reactivity of IC; ● Isolation and characterization of CIC described from the test; all tests described are broadly positive in various human diseases except conglutinin binding tests (?) due to reaction to short lived C3bi fixed complexes.

TABLE # I. ANALYSIS OF METHODS FOR DETECTION OF CIRCULATING IMMUNE COMPLEXES(CIC):
(Seven selected antigen non-specific methods)

d. Tests that influence agglutination, C activation or cell--mediated reactions by binding via Fcr:

i) ADCC inhibition (87),

ii) Red cell rosette inhibition (175),

iii) Platelet aggregation test (135, 154),

iv) Latex agglutination antiglobulin test (89, 104),

v) Complement consumption test (133),

vi) Anti-complementary activity (82).

All these methods (except iv) require heat inactivation of test sera as a part of decomplementation. This procedure itself causes aggregation of Ig and influences the tests. Unless the tests are modified to use non-heat activated sera they are not reliable. Standardization of these tests is difficult due to the presence of other biologic material in test sera which could exert positive or negative influences.

Selected aspects of different tests in each category are given in Table 1. From this table it will be noted that each test detects CIC of different biologic characteristics. For example, Raji assay detects only IgG bound C_3 fixed complexes, C1q-BA detects both IgG and IgM bound complexes but complement activation has to be via the classical pathway only, and ADCC inhibition detects non-complement fixed IgG type complexes, etc. Knowledge of limitations and advantages of each test therefore becomes critical when one wants to select a test for investigation of a particular disease. In this respect, therefore, no one single test would provide an answer as CIC patterns in terms of ab class or ab/ag ratio will change with disease. Therefore it would be ideal to use a combination of more than one test. A WHO organized multicenter trial was organized in 1978 to determine the sensitivity and usefulness

of different tests in different pathological sera (97). Out of 18 different tests only the six below were found to be useful:

1. Raji cell RIA
2. Clq-BA (fluid phase)
3. Clq-solid phase (SP)
4. Conglutinin SP
5. Monoclonal RF inhibition
6. Platelet aggregation test.

These were recommended for investigative purposes with use of more than one test and selected knowledge of tests in a given disorder. The Raji cell RIA and the Clq-BA are the most widely used because of their sensitivity, reproducibility, and wide spectrum coverage of ICS. Background information on these two tests is given below.

Clq-BA:

This is the first method described for CIC detection by Agnello et al in 1970 (6a). This was based on precipitation of IC in the presence of excess of Clq. It was not very sensitive, was only qualitative, and soon was followed by use of quantitative assay by Nydegger et al (142) and later modified to use radiolabelled ^{125}I Clq without heat inactivation of serum (231). This involved precipitation of IC bound to ^{125}I Clq by 2-3.5% PEG precipitation. As modified it detects IgG and IgM bearing complexes, and was then widely used in different laboratories due to simplicity of the technique. Major disadvantages were due to binding of ^{125}I Clq to non-IC materials and subsequent precipitation by PEG. Modifications of the Clq-BA method thereafter were developed (8, 86, 176). Solid phase Clq assay was developed later (69) and is one of

the most sensitive tests currently available without the disadvantages of the fluid phase of Clq-BA, but detects only IgG bound complexes.

Raji Cell RIA:

Historical background of the development of this test is very interesting. Dr. Frank J. Dixon of Scripp's Clinic, U.S.A., went to Bangkok in 1971 as the head of a field study of Dengue fever, for his interest in viral immunopathology. Searching for circulating immune complexes in patients with severe haemorrhagic shock, he obtained suggestive evidence for the presence of such complexes on the surface of circulating leucocytes. On his return to Scripp's, he extended this observation in the growth of Raji cell assay for CIC (134).

It was observed by his group and others that human B lymphocytes and certain lymphoblastoid cell lines would bind aggregated IgG or antigen complexed Ig via Fcr or complement receptors Nussenzweig, 1974; Theofilopoulos et al, 1974a; Dickler, 1976). Searching for a B cell line without surface IgG, they found that a cell line derived from a Burkitt's lymphoma (Raji cells) would be most suitable (Theofilopoulos et al, 1974a, b) for the test. Raji cells contained very avid C_{3b}, C_{3d} and other complement receptors (C_{5a}, Clq) as well as IgG-Fcr of poor avidity (193) but no surface Ig. The Raji line was used to detect IC by testing them with heat aggregated IgG, in vitro complexes, and later, pathological sera (199). It was established that C_{3b} and C_{3d} receptors on Raji cells are more avid in in vitro binding to IC than Fcr and Clq receptors. The sensitivity of Raji assay was better than other tests such as anticomplementary activity (226), and conglutinin binding assay (45), and later became one of the most sensitive tests. Drawbacks were (i) maintenance of a cell line and, (ii) reactions with antilymphocytic

antibodies (2, 9). These have been fully described in Chapter III, Section 3 of this thesis.

2. Pitfalls of CIC Methods

a. Storage of samples

Storage of serum aliquots at a 4°C or lower temperature would cause self aggregation of IgG with other Ig molecules in vitro with or without complement activation and would be influenced in the same way by repeated freezing and thawing. Since all of the antigen non-specific tests are dependent on the determination of complement or antibody molecules in the IC they would be affected by these changes and more so if the tests are sensitive to AHG or complements.

Storage at -20°C was better than -4°C and best at -70°C and specimens stored at a temperature below -70°C are good for all tests up to an average of eight weeks but Raji tests have been shown to be equally reproducible after storage of several months (226, 229). On the other hand, PEG dependent tests are greatly affected by storage beyond seven to twelve days (40).

b. Heating of serum samples

Heating of serum is necessary for anticomplementary activity dependent tests (82, 133, 175), some forms of Clq inhibition tests (176), and ADCC inhibition tests (87). Heating causes aggregation of IgG molecules in sera giving false positives and is therefore generally avoided.

c. Standardization of CIC assays with aggregated human Ig

Most of the CIC assays are standardized with heat aggregated human IgG (AHG) as a model for in vitro IC and results are expressed in AHG equivalent units. This practice originated from the fact that AHG is (i) simple to prepare, (ii) readily available commercially, (iii) when

heat aggregated it would assume the property of complement activation or RF factor binding (144, 208), unlike that in monomeric forms of IgG, subserving some of the properties of in vivo IgG in the complexed form with antigen (39) and, (iv) standard curves prepared with different concentrations of AHG would allow standardization and quantitation of day-to-day results.

It was soon realized that these expectations were not met with AHG and this was confirmed by the first WHO study (97) that AHG preparations of different batches behaved differently in different tests. It was very unstable and aggregate size varied from storage and day-to-day handling (180). Reproducibility of AHG standard curves was poor and at present it should not be used as an ideal standard.

The other serious drawback of AHG standard curves was that it expressed the concentration of AHG, not the size of aggregate, whereas binding of AHG to different ligands or cell receptors depends on its lattice structure and aggregate size (11, 39, 208). Therefore due to change of sizes in AHG in handling and storage, the AHG standard curve would vary in shape and expression by concentration and would indicate poor correlation with lattice size of the in vitro complexes.

Stabilized forms of AHG have been demonstrated by adding BSA (90). Monitoring of aggregate size by non-radiolabelled procedures has also been described (116) but, at present, data on these stable AHG preparations are limited. Suitability and selectivity of such stable AHG preparations are the subjects of a second WHO study (216). Until this problem is delineated, comparison of CIC results from different laboratories on AHG equivalents is not very meaningful.

Presently some investigators would include a batch of reference standard normal sera in each given test. Assuming a normal Gaussian distribution of CICs, the values above the mean $\bar{X} \pm 2$ SD would be taken as abnormal results by the test (205).

Other major drawbacks of the individual tests are given in Table 1. Except for a few (21, 89), most of the tests are influenced by in vitro aggregation of IgG either via Fcr binding or by activation of complements.

No tests are available at present which can detect IgD/IgE containing complexes and few tests have been developed to detect IgA containing complexes (64, 102), but the differentiation between high serum levels of IgA and IgA-containing complexes by these tests was not clear.

3. Isolation of Antigens From CIC Assays

IC are formed by non-covalent bonds between the ag and ab. This could be isolated by breaking the non-covalent bonds by (i) acid or alkali treatment (208), (ii) enzyme digestion (66) and, (iii) acid or alkali elution followed by affinity chromatography or gel filtration (70).

From the established IC assays, procedures to isolate IC are derived from UC, PEG precipitations (16, 21, 40), conglutinin BA (26), and Raji RIA (196). Complex biochemical procedures with gel chromatography and immunoprecipitation, etc., are needed for subsequent isolation of the critical antigens from other non-specific molecules. By Raji RIA, specific antigens have been isolated from CICs in animal models and subsequent ab or ag injection has been done for immunization (196). If successful, ag isolation from CIC would ultimately lead to

the fulfillment of identification of idiopathic IC-mediated disease as well as vaccine production. However, on the whole these efforts are extremely complicated due to the presence of co-existing antigen antibody molecules of different nature.

4. Clinical Application of CIC Assays

The ultimate objective of CIC detection is to demonstrate its validity in clinical perspectives and in understanding the immunopathology of IC-mediated diseases namely; correlation with disease activity, relation of CIC with tissue deposits and isolation of antigen(s) from CIC. With these objectives, CIC have been detected in various disorders and reported in recent literature (141, 194). It is not possible to cover all aspects of these results but suffice it here to mention that it has produced conflicting results, causing some misunderstanding regarding the validity of CIC research in the immunological sciences. The author would only like to describe one clinical situation where CIC research has been applied extensively and will try to present, in true perspectives, the difficulties in application of these research results in clinical state as well as the causes of misunderstandings based on their conclusions.

Glomerulonephritis (GN) is one of the best demonstrated forms of IC-mediated disease in experimental animals and man (43, 55). Kinetics of IC formation, clearance and deposits in animal models are well known (43, 124) and hence CIC detection would form a feasible application in such a condition. This was, therefore, actually done in various centres, with great enthusiasm. The aim that CIC levels would provide an objective measure to monitor disease activity in GN patients, particularly in idiopathic GN and isolation of antigens from CICs, would

provide an answer to the etiology of these disorders. To the dismay of many investigators, CICs were not usually detected where they were supposed to be abundant by way of tissue deposits of ICs (eg. in idiopathic membranous GN) (40, 162, 205, 226), whereas they were present where there were no pathological demonstrations of renal IC deposits (eg. in nil lesion disease) (18, 101, 158).

On the other hand, as expected in proliferative forms of idiopathic GN and in SLE nephritis, CIC levels and renal tissue deposits of IC correlated well (40, 145, 146, 206, 226). Some investigators found CIC levels were an even better indicator of disease activity than anti-DNA levels in SLE patients (23), while others found good correlation between disease activity, CIC levels and anti-DNA antibodies (3, 36, 68, 103) in the same disease.

To the practicing clinician, detection of CIC was not helpful to monitor disease activity in most of the forms of idiopathic GN although greater than 80% would be positive for IC deposits by IF or EM studies in the kidney biopsies. To the researcher, it did provide an alternative approach for the understanding of pathogenesis of IC mediated renal diseases, namely: that in many of them IC-mediated renal disease would occur without CICs, accounting for local formation of ICs in the kidney due to alteration of local conditions and formation of antibodies in circulation (in situ IC formation); or there could be possible fluctuations in the levels of CIC and glomerulonephritis may result from recurrent brief and not easily detected bouts of IC deposition (218).

Coming back to the lupus nephritis and relation with CIC, it was found that after showing a good correlation with CIC levels and disease activity, various investigators then attempted to isolate and character-

ize the CICs in terms of size, clearance and DNA content of the complexes. A recent NIH study has confirmed poor clearance of CIC by RE systems in SLE patients over controls (52). Several independent workers have shown presence of small and intermediate size complexes in SLE patients (21, 103, 155, 206) and others have characterized a relationship between the size of CICs and the type of renal lesions (206). However, except for two (5, 21), most of these investigators failed to demonstrate DNA containing complexes in CICs from SLE sera (6b, 61, 79, 182, 183). These findings taken together, at this point, would mean that occurrence of CIC in SLE has no disease specificity and could be an epiphenomenon.

Experimental work on NZB and other strains of mice by Izui et al (1980) would attest to the above fact as they did not find DNA-anti-DNA containing complexes (78), but found GP-70 - anti-GP-70 complexes where GP-70 represented a specific viral envelope protein (198). Extrapolation of these animal results would suggest; (i) in human SLE patients do not form DNA-anti-DNA complexes in circulation or, once formed, are rapidly cleared from circulation and by virtue of its avidity would localize to kidney tissue and mediate injury. CIC levels would therefore be non-specific for DNA containing complexes or; (ii) our knowledge for isolation of DNA containing complexes may not be perfect at present. Experimental conditions may lead to changes in physico-chemical conditions which lead to denaturation of existing DNA in complexes. In a recent meeting of the International Congress of Rheumatology in Paris, Steinman presented the pitfalls of experimental conditions of DNA isolation techniques from CICs. It was emphasized that plasma samples had a better chance of yielding DNA than serum samples (182) which most inves-

tigators used except the two who were successful. One of them used plasma (21), the other, cryoprecipitates (5).

The difficulties in the isolation and identification of ag with other unknown ag-ab system(s) should be anticipated. Understanding the limitations and scope of individual systems, and proper knowledge in research conditions, are evidently required for pursuing further research in CIC fields.

The main task of this study was to explore the possibilities of whether evaluation of CIC levels in certain selected clinical conditions would help us to understand CIC-mediated immunopathology in these disorders. Data is presented in studies of cystic fibrosis (CF), multiple sclerosis (MS), and hemodialysis patients. Detailed review of the literature and objectives in these diseases are given separately for each of them.

Considering the various limitations of different CIC methods, the project also modified and developed the Raji cell radioimmunoassay (Raji RIA), one of the most sensitive methods available, and compared data obtained with a combination of results with another sensitive method of CIC detection: i.e. C1q binding activity (C1q-BA).

Chapter II: Methodology

I. CIC Methods

A. Raji cell radioimmunoassay (Raji-RIA) for the detection of CIC:

1. Raji cell line conditions

Raji cell seeds were initially obtained from Dr. Longenecker in the Department of Immunology, University of Alberta and later from Dr. A.N. Theofilopoulos, Scripp's Clinic, La Jolla, California, and were maintained in continuous culture in Eagles minimum essential medium (MEM) (Appendix). Cells were cultured at a density of 2×10^5 cells/ml in tissue culture flasks at 37°C without shaking. Receptors for Fc, C_{3b}, and C_{3d} are expressed equally well throughout the cell cycle (195). However, cells used in the assay were obtained 72 hours after initiation of culture.

2. Radiolabelling of anti-human IgG

IgG fractions of rabbit anti-human IgG were obtained from Cappel Lab, P.A., U.S.A., and were radiolabelled with ^{125}I by following the method of McConahey and Dixon (117). Our approach in radiolabelling in comparison to the original description from Scripp's Clinic is given in Table 2.

3. RIA test procedures

This was done by adapting to the original method described by Theofilopoulos and Dixon (195), with certain modifications in standardization procedure without AHG.

i) Raji cells removed from 72 hour culture (cell density 1×10^6 cells/ml) were mixed with two drops of trypan blue and cell number and cell viability were assessed with a

TABLE 2

^{125}I Radiolabelling of (Rabbit) Anti-human IgG

		Method of Theofilopoulos et al (199) Scripps Clinic	Our method
1.	Method	Chloramine T McConahey and Dixon (1966) (117)	same
2.	Exposure time to Chloramine T.	> 1 minute	< 15 seconds
	Av. Iodine uptake %	> 65	> 65
3.	Final process of purifying labelled protein	Dialysis 12-24 hrs	Column separation
4.	Dose of ^{125}I sodium iodide	3000 μCi	1000 μCi
5.	Antibody specific protein of stock Ig	5 mgm/ml	5 mgm/ml
6.	Final antibody protein concentration in labelled antiserum	1 mgm/ml	1 mgm/ml
7.	Sp. Activity	0.3 Ci/gm	166 μCi/mgm
8.	BSA (RIA grade)	none added	2 - 2.5% added
9.	Labelling efficiency	--	> 95% by TCA precipitable protein

hemocytometer. Cells with viability of 98% or greater were used for the assay.

ii) Raji cells were then washed x 3 times in wash medium (RPMI-1640 with no added protein - Appendix 2), by centrifugation at 1500 rpm (500 g) x 10 minutes at 4°C.

iii) Aliquots of 2 x 10^6 cells (100 μl) of washed cells were placed in 1.0 ml Fisher tubes and 1 ml of wash medium was added to each tube and centrifuged 1500 rpm (500 g) x 10 minutes at 4°C.

iv) After centrifugation, supernatants were discarded, and cell pellets (2 x 10^6 Raji cells) resuspended in 50 μl of wash medium. To this 25 μl of test serum (diluted 1:4 in 0.15 M NaCl saline) were added.

v) Tubes were incubated at 37°C for 45 minutes with intermittent gentle shaking by hand (every 5 to 10 minutes).

vi) At the end of this incubation period cells were washed x 3 with wash medium. For the first wash, 1 ml of wash medium was added and cells centrifuged at 1500 rpm (500 g) for 10 minutes at 4°C. Supernatants were aspirated and cell pellets resuspended in 200 μl of wash medium with gentle mixing with a Pasteur pipette and subsequently 1 ml of wash medium added. Resuspended cells were then centrifuged as in the first wash. This step was repeated for the third wash.

vii) After the final wash, cell pellets were resuspended in 50 μl of wash medium containing 1% BSA (RIA grade, Sigma Lab, Cat No. A 4378) and 50 μl appropriate amount of ^{125}I rabbit - anti-human IgG* (determined for each batch of antibody by a

critical titration between a highly positive sera and normal sera, giving a difference of uptake greater than 7-8 times of normal sera) diluted in wash medium containing 1% BSA was added and mixed thoroughly. An incubation period of 30 minutes at 4°C was carried out with the mixture, with intermittent gentle shaking by hand at 5-10 minute intervals.

viii) At the end of the incubation period cells were washed in medium containing 1% BSA by following similar procedures as in step (vi). After the final wash the supernatants were aspirated close to cells, and pellets were counted in a gamma counter. Final results were calculated from the duplicates of each serum.

4. Standardization

AHG was not used as a standard in this assay, as originally described(199). Instead 10 NHS were included in each experiment to find out the optimum binding of ^{125}I anti-human IgG. The CPM values of the mean ($\bar{X}$) and SD of these values were calculated and CPM above the $\bar{X}$ + 2 SD values of the reference NHS on the day of experiment was considered abnormal or positive results. For quality control and ensuring reproducibility of day-to-day results, a serial dilution of highly positive SLE sera (LJ) was run. At a critical dilution (1/100) the sera were found to be just above the 2 SD of $\bar{X}$ of 10 reference NHS. This was used as an additional index to ensure standardization and reproducibility of test results done on different days. Details of this modification and standardization are given in Chapter III, Section I of this thesis. Raji RIA results are expressed as SD above the normal mean ($\bar{X}$).

5. Clq-binding assay (Clq-binding activity by PEG dependent, fluid phase assay) - Clq-BA

This was done by following the methods of Zubler and Lambert (229) as already established in our laboratory as a routine CIC assay (84). Briefly, human Clq was isolated and purified from pooled normal human sera (NHS) by binding to calf thymus DNA, DNAase digestion and Sephadex G200 column separation (Jones and Cummings 1977). Purified Clq was checked with anti-human Clq in radial immunodiffusion and immunoelectrophoresis and also against anti-whole human sera. This formed a single line of precipitation. Purified human Clq was radiolabelled with ^{125}I by the lactoperoxidase method (130).

50 µl of test serum were mixed with 100 µl of EDTA (to inactivate endogenous free Clq) at 37°C x 30 minutes. Negative and positive controls were also put in the same way by NHS from healthy donors and positive sera from SLE, RA and other patients. Solutions of AHG in PBS of known concentrations were also added as positive controls.

After this incubation, tubes were placed in ice baths and 50 µl of ^{125}I Clq added and immediately thereafter, 1 ml of PEG (3.5%, MW 6000 daltons) solution was added to these mixtures and kept at 4°C x 1 hour. Two TCA control tubes were prepared by mixing 50 µl of ^{125}I Clq solution and 150 µl of serum and 1 ml of 20% TCA. After 1 hour all tubes were centrifuged at 1500 g (3000 rpm) x 20 minutes at 4°C. Supernatants were discarded and radioactivity of the pellets was measured.

Test results were expressed as % of ^{125}I-Clq prepared by PEG as compared to total CPM precipitated in the TCA control tubes. Each test was run in duplicate and the mean of duplicate tests represents Clq binding activity, Clq-BA.

Normal results determined in healthy donors were found to be 6.0 ± 2.2 as $\bar{X}$ 1 SD. Results $\geq \bar{X} + 2$ SD were considered abnormal, i.e. values $\geq$ 11.1% binding were taken as positive results.

6. Handling of Serum Samples

All blood samples were allowed to clot at room temperature for one hour, centrifuged at 400 G for 10 minutes, divided into 0.5 ml aliquots and stored at -70°C. Aliquots of serum were thawed once for assays on the same day; they were assayed within 2 months of collection.

7. Statistical Analysis

Student's "t" test, Chi square, and Fischer exact tests were used for statistical evaluation of the data. They have been described in individual chapters where such applications were made.

Chapter III: Developmental Aspects of Raji Assay

1. Raji Cell RIA - Standardization on L.J. Serum

We have introduced a new method for standardization of the Raji-RIA assay, by referring to a standard curve prepared daily. Previously, the standard curve was obtained by referring to uptake of ^{125}I anti-human IgG (either in percent or by CPM) to 13-14 serial samples of normal human serum containing heat aggregated gamma globulin (AHG + NHS), where the quantity of AHG added to such samples varied from 1 μ gm/ml to 4-8 mgm/ml. A sample of pooled normal human sera was run every day to find out the corresponding background uptake of ^{125}I anti-human IgG, mediated via Fc receptors on Raji cells and 7s IgG on NHS.

We found that, by referring our results to such AHG standard curve, the lowest value of CIC detected was 4 μg/ml and highest > 4 mgm/ml of AHG equivalent. In 30 normal blood bank donors the mean + 2S.D. value was found to be 33 μg/ml of AHG. Values greater than these were considered abnormal. A representative AHG standard curve is shown in Fig 1.

Unfortunately we noticed the AHG standard curve had poor reproducibility (due to instability on storage) and one experiment could not be compared to another. We, therefore, established a modified way of standardizing the assay without the use of AHG.

Our goal was to differentiate sera containing CIC from normal human sera. We took a highly positive serum from an SLE patient and ran it in several dilutions each day in parallel with 10 normal human sera (NHS). Uptake of ^{125}I anti-human IgG, above the mean + 2 SD values of the 8-10 NHS samples, is considered abnormal and expressed in SD units by the

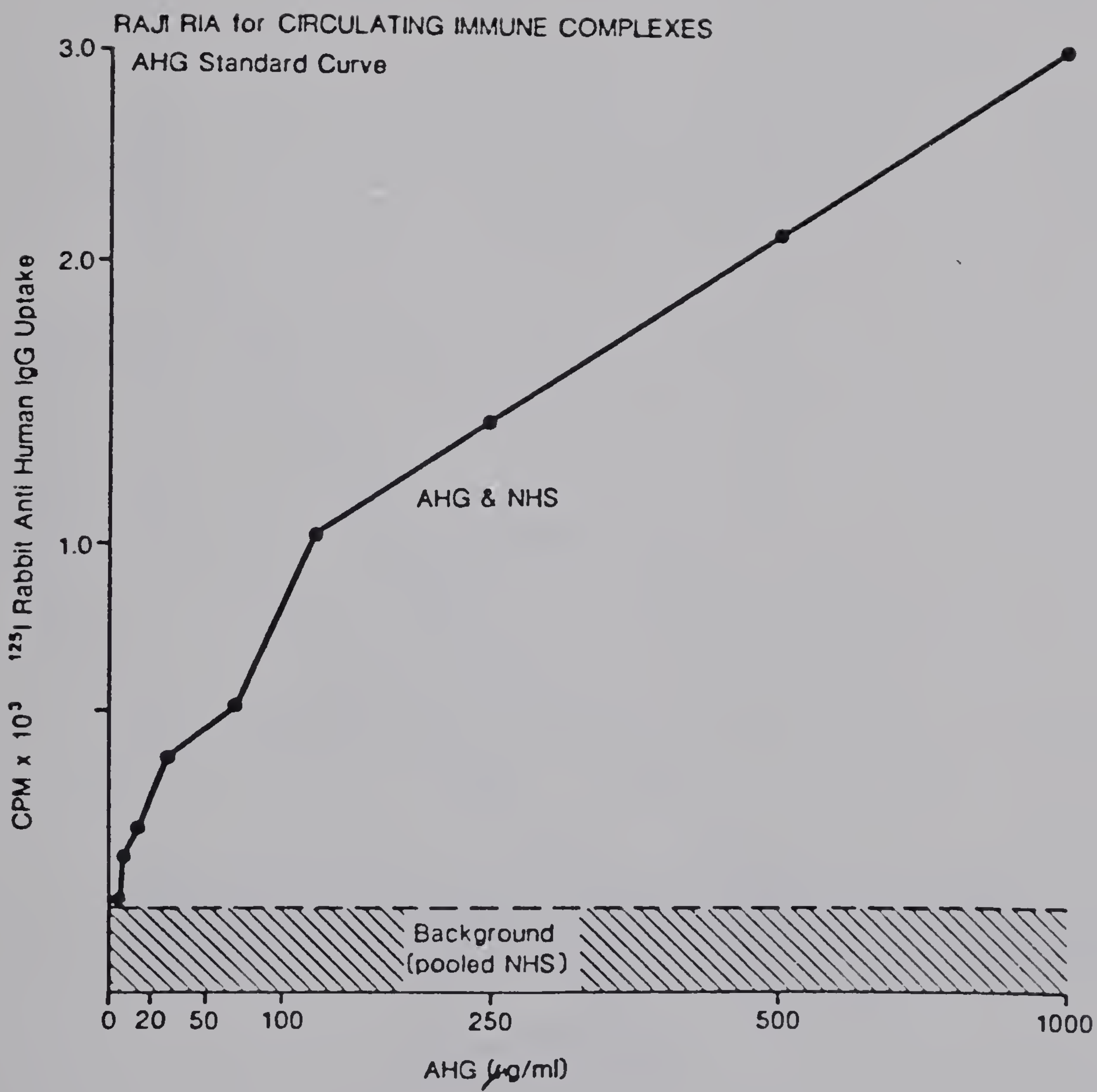

Fig. 1. Raji RIA for circulating immune complexes.

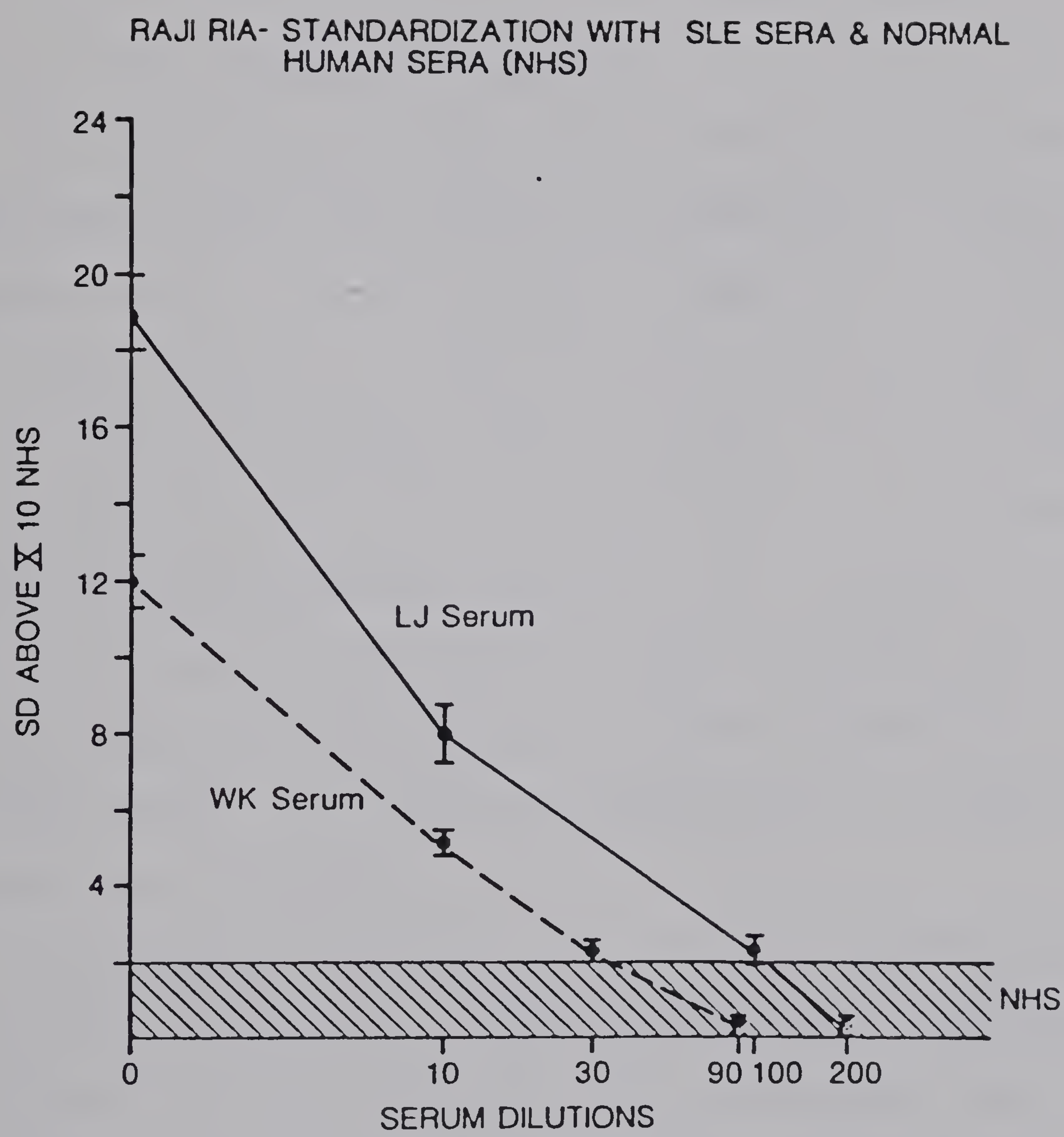

Fig. 2. Raji-RIA standardization with SLE sera and normal human sera (NHS).

following calculation:

Test Score

$$= \frac{\text{CPM observed in test sample - mean CPM of 8-10 NHS}}{\text{Standard deviation of mean CPM of 8-10 NHS}}$$

$= > 2$ SD = abnormal (or positive)

As shown in Figure 2, this batch of serum (L.J.) at 1:100 dilution consistently gives values 2 SD above the mean of 10 NHS even with repeated testing. Therefore, instead of AHG, we aliquoted small amounts of LJ sera and ran it in serial dilutions in each experiment, together with 8-10 NHS at dilution of 1/100 L.J. serum was always just above 2 SD of the mean CPM of NHS sera run at the same time. This allows quality control and standardization, and corrects for variation in the system.

This serum was also positive in other CIC assays, e.g. Clq and BA and was negtive for antilympohocytic antibodies against Raji cell target in ADCC and CDC. Also, serum IgG concentration of L.J. sera was within normal range (1190 mg/dl).

This method of standardization can be done with other highly positive serum from patients with SLE or other diseases containing in vivo complexes. A critical dilution to give a reading just above 2 SD of reference NHS would then be determined. Another SLE sera WK used in such a way is shown in Fig. 2.

In Fig. 3, we have shown two AHG standard curves of ^{125}I rabbit anti-human IgG uptake (Y) vs concentration of AHG (X), and the slopes or regression line Y on X (Y = a + bX). Values of different AHG standard curves (done with same batch of AHG) are given in Table 3. They show very wide variation (coefficient of variation = 50.3%); in contrast

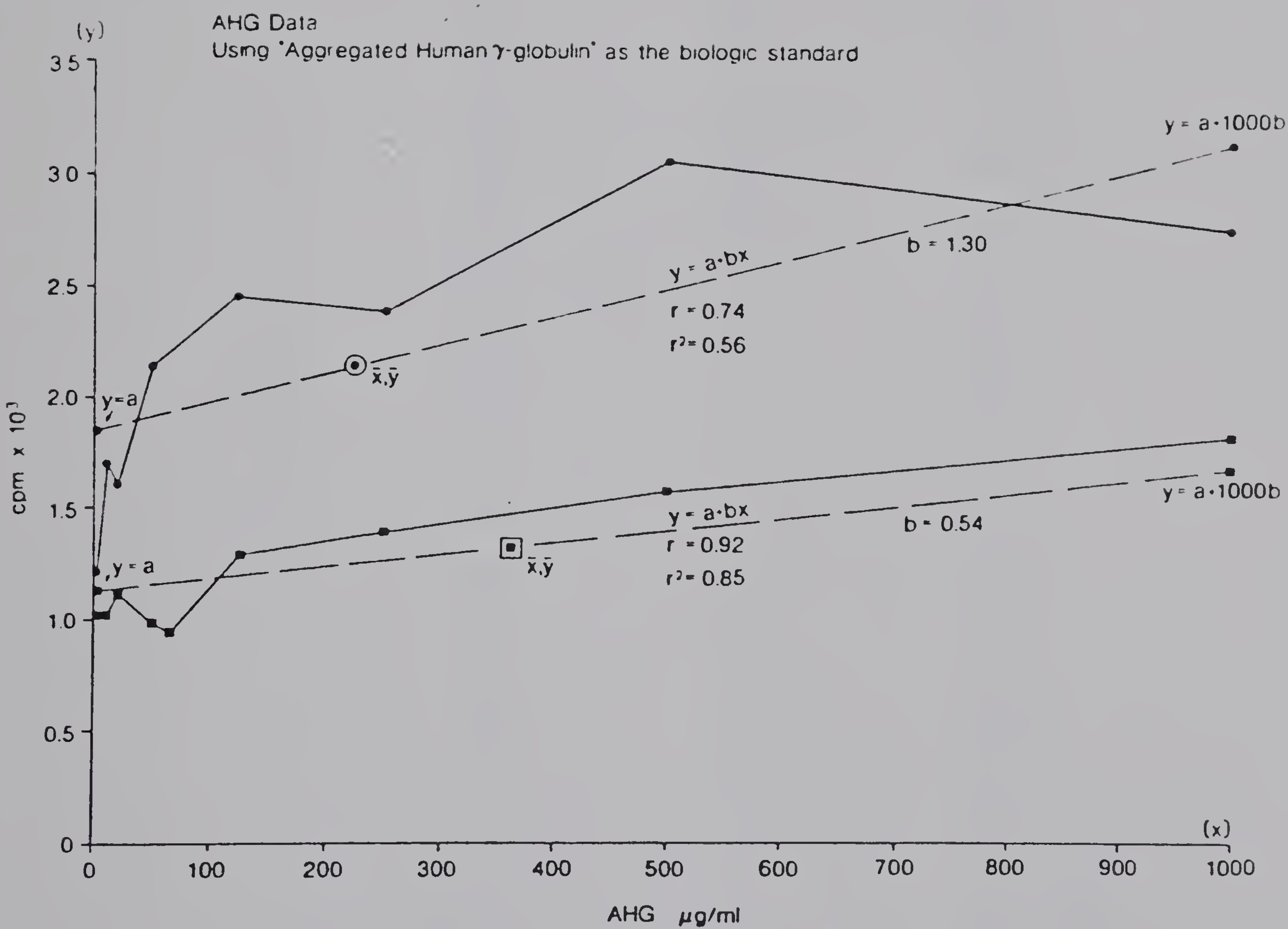

Fig. 3. AHG data using "aggregated human gammaglobulin" as the biologic standard.

TABLE 3

AHG STANDARD CURVE

Correlation with ^{125}I Anti-Human IgG Uptake

	r	r^2	b
1.	54	30	0.27
2.	67	45	0.29
3.	72	52	0.30
4.	74	56	1.30
5.	75	57	0.85
6.	80	64	0.87
7.	80	65	0.114
8.	84	71	0.57
9.	88	78	0.81
10.	89	80	1.29
11.	91	83	0.73
12.	91	84	0.74
13.	92	85	0.54
14.	93	88	0.78
15.	96	94	0.74
			$\bar{X}$ = 0.68
			SD = 0.34
			SE = 0.08
			CV = 50.29%

r = Correlation Coefficient

r^2 = Coefficient of Determination

b = Slope of regression Line Y on X

CV = Coefficient of Variation

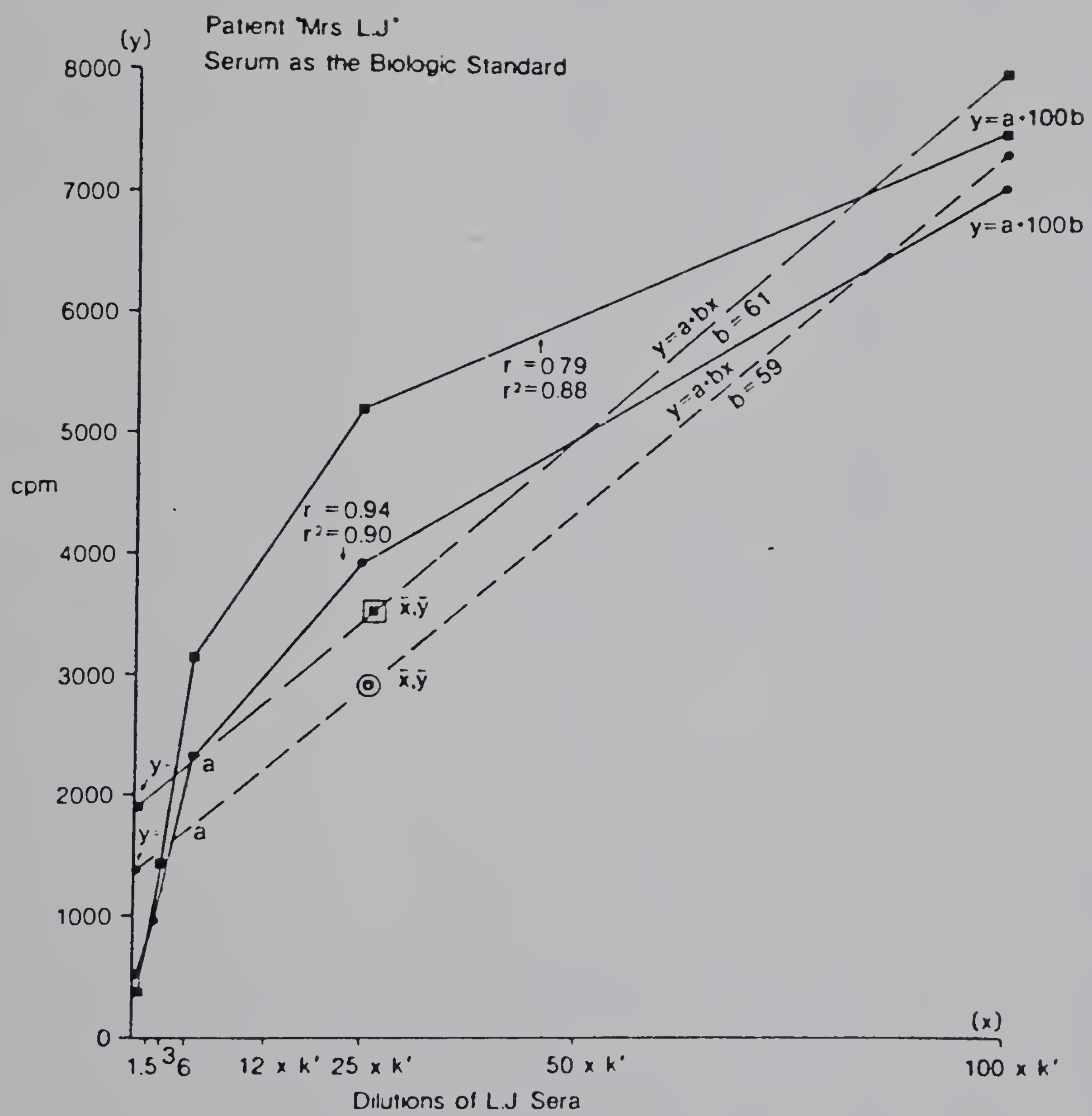

Fig. 4. Patient "Mrs. L.J."

TABLE 4

L.J. Serum Titration

Correlation with <125 > I Anti-Human IgG Uptake

	r	r^2	b
1.	83	70	16.73
2.	90	81	15.88
3.	91	83	14.20
4.	91	84	14.97
5.	93	83	18.68
6.	93	88	14.45
7.	95	91	17.58

$\bar{X}$ = 16.07

SD = 1.67

SE = 0.63

CV = 10.4%

r = Correlation Coefficient

r^2 = Coefficient of Determination

b = Slope of Regression Line Y on X

CV = Coefficient of Variation

TABLE 5

L.J. Standard Curve in % Uptake of ^{125}I Anti-HIgG to Raji Cells

From 15 Consective Experiments

Expt. No.	L.J. 1/10 % Binding	*CF 1/10	L.J. 1/100 % Binding	*CF 1/100	+NHS $\bar{X}$ % Binding	*CF (for each day)
1.	5.28	0.98	3.29	0.88	2.46	0.81
2.	5.02	1.03	3.96	0.74	2.66	0.75
3.	3.25	1.59	2.03	1.43	1.45	1.38
4.	4.03	1.28	2.52	1.15	1.49	1.34
5.	4.89	1.06	2.75	1.05	2.15	0.93
6.	4.86	1.06	2.93	0.99	2.03	0.99
7.	5.79	0.89	3.14	0.92	2.33	0.86
8.	5.44	0.95	2.90	1.00	2.04	0.98
9.	4.29	1.20	2.02	1.44	1.50	1.34
10.	5.23	0.99	2.88	1.01	1.86	1.08
11.	6.53	0.79	3.32	0.87	1.92	1.04
12.	5.10	1.01	2.65	1.09	1.90	1.05
13.	6.10	0.85	3.06	0.95	2.26	0.88
14.	5.94	0.87	3.21	0.90	1.98	1.01
15.	6.26	0.83	3.11	0.93	2.17	0.92

		L.J. 10	L.J 100	$\bar{X}$ NHS
Observed	$\bar{X}$ =	5.19	- 2.91	- 2.01
	SD =	0.87	- 0.49	- 0.35
	CV =	0.16	- 0.16	- 0.17

$$\text{*CF} = \frac{\bar{X}\ 15\ \text{observed}}{\text{observed value}}$$

CV coefficient of variation

\+ NHS Reference normal human sera (n = 10) to determine normal range in each experiment.

* CF Correction factor, determined to check day to day variation (interassay variation) of individual results. This is caluclated for each day from the mean of the ratio of L.J. (1:0) and L.J. (1:100) to the overall mean for these two dilutions. Note that in most of the experiments the ratio is close to 1. The CF of NHS is not used to calculate CF, but is given as an additional check.

TABLE 6
RAJI-RIA: Comparison of Results of Consecutive Experiments on L.J. Sera at the Initial Period and After One Year of Storage, in Terms of ^{125}I Anti-human IgG Uptake or S.D. Units

	Raji RIA Sera				
	L.J. (1/100)			L.J. (1/100): after 1 year+	
Expt No.	% ^{125}I anti* IgG Binding	S.D. Units	Expt No.	% ^{125}I anti IgG Binding	S.D. Units
1.	3.29	2.8	1.	2.81	2.3
2.	3.96	3.2	2.	2.67	2.0
3.	2.03	2.0	3.	2.91	2.3
4.	2.52	2.1	4.	2.96	2.2
5.	2.72	2.2	5.	2.59	2.0
6.	2.93	2.7	6.	3.20	2.6
7.	3.14	2.6	7.	2.85	1.95
8.	2.90	2.5	8.	3.26	2.6
9.	2.02	2.2	9.	2.80	2.3
10.	2.88	2.7	10.	3.24	2.8
11.	3.32	3.0			
12.	2.65	2.8			
13.	3.06	2.4			
14.	3.21	3.8			
15.	3.11	2.6			
X =	2.91	2.64		2.92	2.30
S.D. =	0.49	0.46		0.23	0.28
Coefficient Variation (c.v.) =	16%	17%		9%	12%

* Same data as of column 3, Table No. 5
\+ Done with different batch of anti-human IgG

TABLE 7
Showing inter and intra assay variation of Raji-RIA results in normal and pathological sera, as well as effects of repeated freezing and thawing

	Diagnosis	Raji RIA-SD Units Experiment No. 1	2	3	Mean $\bar{X}$	Standard Deviation S.D.	Coefficient of variation %
A.	Aligquoted Samples thawed once on the day of experiment						
1.	SLE	6.1	5.7	6.1	5.96	0.23	3
2.	RA	5.2	5.6	5.6	5.46	0.23	4
3.	MS	3.2	2.8	2.6	2.86	0.30	10
4.	Thryoiditis	6.9	7.4	6.8	7.03	0.32	4
5.	CF	5.4	6.7	5.1	5.73	0.85	14
6.	SABE	6.9	7.7	7.2	7.26	0.40	5
7.	MS	3.0	3.1	2.8	2.96	0.15	5
8.	BB Donor 1	0.5	0.4	0.4	0.42	0.05	13
9.	"	0.6	0.5	0.6	0.56	0.05	10
10.	Lab Donor	1.64	1.72	1.75	1.7	0.05	3
B.	Samples Thawed on consectuive experiments						
		1st thaw	2nd thaw	3rd thaw			
1.	SABE	5.0	5.0	3.4	4.46	0.92	20
2.	SLE	3.2	2.7	1.7	2.53	0.76	30
3.	MS	2.7	2.3	0.3	1.76	1.28	72
4.	MS	13.1	8.4	3.0	8.16	5.05	291
5.	Melanoma	6.9	0.2	0	2.26	3.92	165
6.	Thyroid	1.6	3.9	0.8	2.1	1.6	76
7.	"	1.06	3.23	0.68	1.65	1.37	83
8.	MS	5.2	7.9	2.7	5.26	2.60	150
C.	Intraassay variation: same sample in one experiment x 3						
	SABE	4.2	4.5	4.6	4.43	0.20	4
	MS	2.3	1.9	2.2	2.13	0.20	9
	BB Donor	1.1	1.3	1.1	1.16	0.15	9
	Lab Donor	0.7	0.7	0.6	0.66	0.05	8

SLE = Systemic lupus erythrematosis.
SABE = Subacute Bact. endocarditis
CF = Cystic fibrosis
MS = Multiple Sclerosis
BB = Blood Bank

curves with L.J. serum showed excellent degree of correlation (Fig. 4 and Table 4) with a coefficient of variation of only 10.4%.

Reproducibility interims of ^{125}I antihuman IgG uptake in consecutive experiments with L.J. sera was found to be very satisfactory (Table 5). A correction factor (CF) is also shown and explained in the table as an additional check.

Based on this modification we evaluated unknown samples, excessive results in SD above normal mean (SD units) instead of AHG equivalent. In Table 6 reproducibility of L.J. sera (Standard) in terms of either ^{125}I antihuman IgG uptake or SD units are shown in comparison to initial experimental results and 12 months later. Representative examples of reproducibility of other test sera in terms of inter or intra assay variations are given in Table 7 as well as effect of repeated freeze thawing.

2. Raji RIA for CIC: Normal Subjects and Diurnal Variations

A total of 181 different samples from three groups of donors were obtained representing a wide cross-ection of normal population in respect to age and sex. No significant differences between the various age and sex groups were noted in regards to CIC by Raji-RIA ($p = > 0.75$ and > 0.50 respectively). Detailed results are given in Fig. 5 and Table 8.

Diurnal variations

In 15 subjects serum samples were collected in the morning (7 to 8 a.m.) and at afternoon (between 5 to 6 p.m.) CIC results are shown in Fig. 6. No significant variations in CIC levels were detected between a.m. and p.m. either by Raji-RIA or C1q BA ($p > 0.50$ by Student's "t"

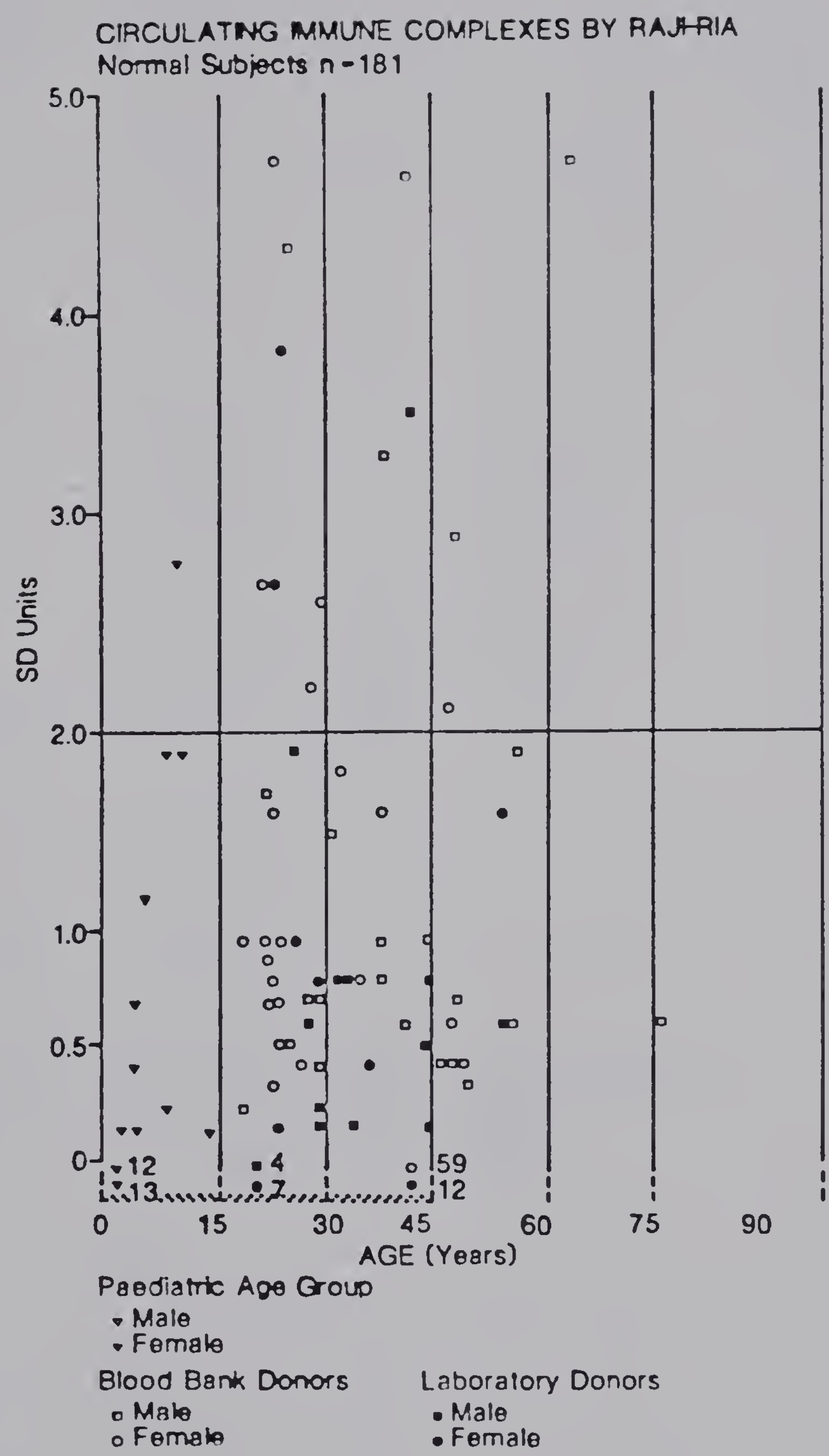

Fig. 5. Raji-RIA results for CIC among normal subjects of different age and sex group. Values are expressed in S.D. units as explained in the text and values around normal mean are indicated in the shaded area at the base of the graph.

TABLE 8

Raji-RIA:CIC in Normal Population (by age and Sex Groups)
N = 181

Age No. groups	A < 20	B 20-29	C 30-39	D 49-40	E 50 or above	Total
Males (N1)	14	31	26	20	18	109+
Positives*	0	1	2	1	1	5+ (5.5%)
Females (N2)	22	28	12	9	1	72x
Positives*	2	6	1	1	0	10 (13.9%)
Total N1 + N2 + N	36	59	38	29	19	181xx
Positives	2	7	3	2	1	15
% positives	5.5	11.9	7.8	6.8	5.2	8.3

* Postives = values of Raji-RIA above 2 S.D. units

xx χ^2 = 1.65, df = 4 not significant (NS)
p = > 0.75

x χ^2 = 2.28, df = r NS
p = > 0.50

+ χ^2 = 1.65, df = 4 NS
p = > 0.75

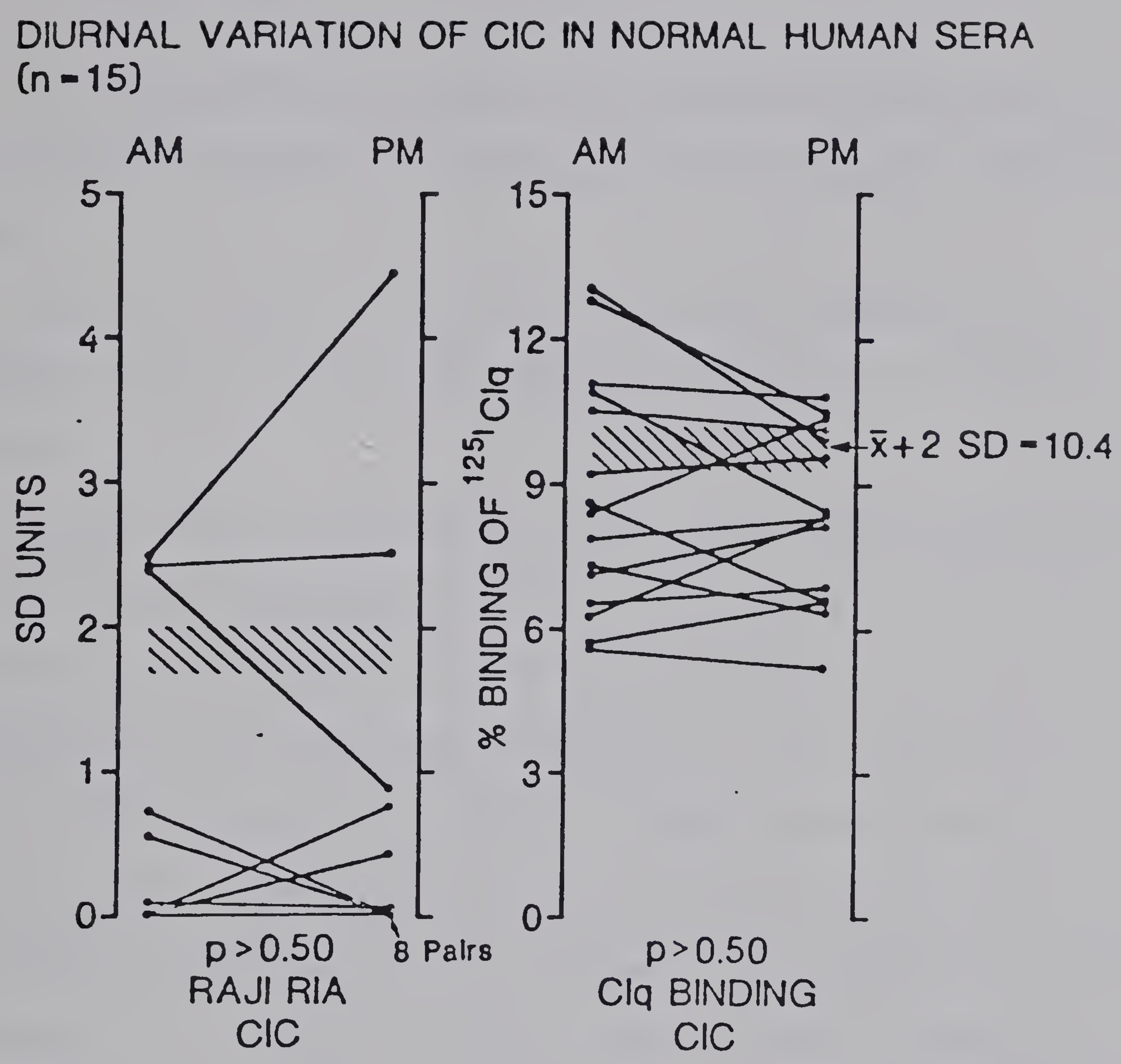

Fig. 6. Diurnal variation of CIC in normal human sera.

test).

1. Blood Bank donors.

2. Laboratory personnel.

3. Donors from the Paediatric group.

3. Comparison of Raji-RIA Results With C1q Binding Activity (C1q--BA) and Bovine Conglutinin Binding Activity in Normal + Pathological Sera.

Raji RIA results in normals and different pathological sera were compared with two other radioimmunoassays for CIC. Results are shown in Table 9.

Higher positive results by Raji-RIA and reduced positivity by Bovine conglutinin assay was noted, as also noted by others (26, 45). Comparison of CIC results in the same disease group by three different methods indicated prevalence of complexes of different biologic characteristics and hence no statistical comparisons were made between the groups tested. The table also points out the need for use of more than one method of CIC and to select the best tests suitable for detection of complexes in a particular disease group, as also recommended by the WHO Study, 1978. (97).

Our results regarding prevalence of CIC were similar to that already reported in the literature except in the areas of multiple sclerosis (MS) and cystic fibrosis (CF). Detailed discussions regarding MS and CF studies are done in Chapter IV and VI respectively.

4. In Vitro Prepared Immune Complex: Detection and Isolation by Raji Assay

In vitro IC was prepared in four times antigen excess with ^{125}I-labelled bovine serum albumin (BSA) and IgG fraction of rabbit anti-BSA

TABLE 9
Prevalence of CIC in Normal and Pathological Sera
by 3 Different Radioimmune Assays

Groups	RAJI-RIA			C1q-BA		Conglutinin BA		Total
	n	+ve	%	+ve	%	+ve	%	Sera
1. Normals (Blood Bank donors 90 subjects	90	8	8.8	4	4.4	7	7.7	90 normals
2. Systemic lupus erythrem-atosus (SLE) 30 patients	102	97	95	34	33.3	6	5.8	
3. Rheumatoid Arthritis (RA) 58 patients	72	40	55.5	45	62.5	9	12.5	
4. Glomerulo-nephritis GN (50 patients)	50	16	32	14	28	8	16	
5. Renal Transplants (33 patients)	84	12	14.2	19	22.6	11	13	
6. Cystic fibrosis(CF) 48 patients	48	18	37.5	5	10.4	0/22xx	xx	
7. Multiple sclerosis (MS) 254 patients	272	80	29.4	40	14.7	12	4.4	
8. Melanoma 20 patients	114	6	5.2	17	14.9	0	0	Patho-logical 742
Total+		269	36.2+	174	23.5+	46	7.4+	

+ve value represents > $\bar{X}$ + 2 SD of normal results in each test
XX only done in 22 samples
\+ Total and % of pathological sera only.

(custom prepared against the same batch of BSA by Cappel Lab, Lot 13687). Detailed steps of these preparations are given in Appendix 3.

Prepared IC were tested in Raji RIA, after incubation with NHS as a source of complement (37°C x 30 minutes). Other controls were set up with the same NHS minus the complexes, ^{125}I BSA and anti-BSA respectively. Representative results are shown in Figure 7. They show BSA-anti-BSA complexes with complement are best detected by Raji assay.

Isolation and characterization of ^{125}BSA from ^{125}BSA-anti-BSA complexes were made by incubating 30 x 10^6 Raji cells with the in vitro prepared IC at 37°C x 45 minutes followed by acid elution at a pH of 2.9 - 3.2 with isotonic citrate buffer following the methods described by Theofilopoulos et al (196). Details are given in Appendix 3. Raji eluate was then subjected to polyacrylamide gel electrophoresis in SDS (SDS-PAGE) as described in Appendix 3. A number of controls were run simultaneously, which consisted of ^{125}BSA alone, anti-BSA, NHS (used as a source of complement), and washing of Raji cells with acid buffer without any IC incubations (to check for Raji membrane proteins isolated during the procedure of elution). Several experiments were done with good isolation of ^{125}BSA from the BSA-anti-BSA in vitro complexes. A representative result is given in Fig. 8 together with controls. Here gel columns were cut into sections and direct counts were obtained in a gamma counter.

The SDS-PAGE part of the experiments were done under guidance and supervision from Drs. T. Nihei and D.L.J. Tyrrell.

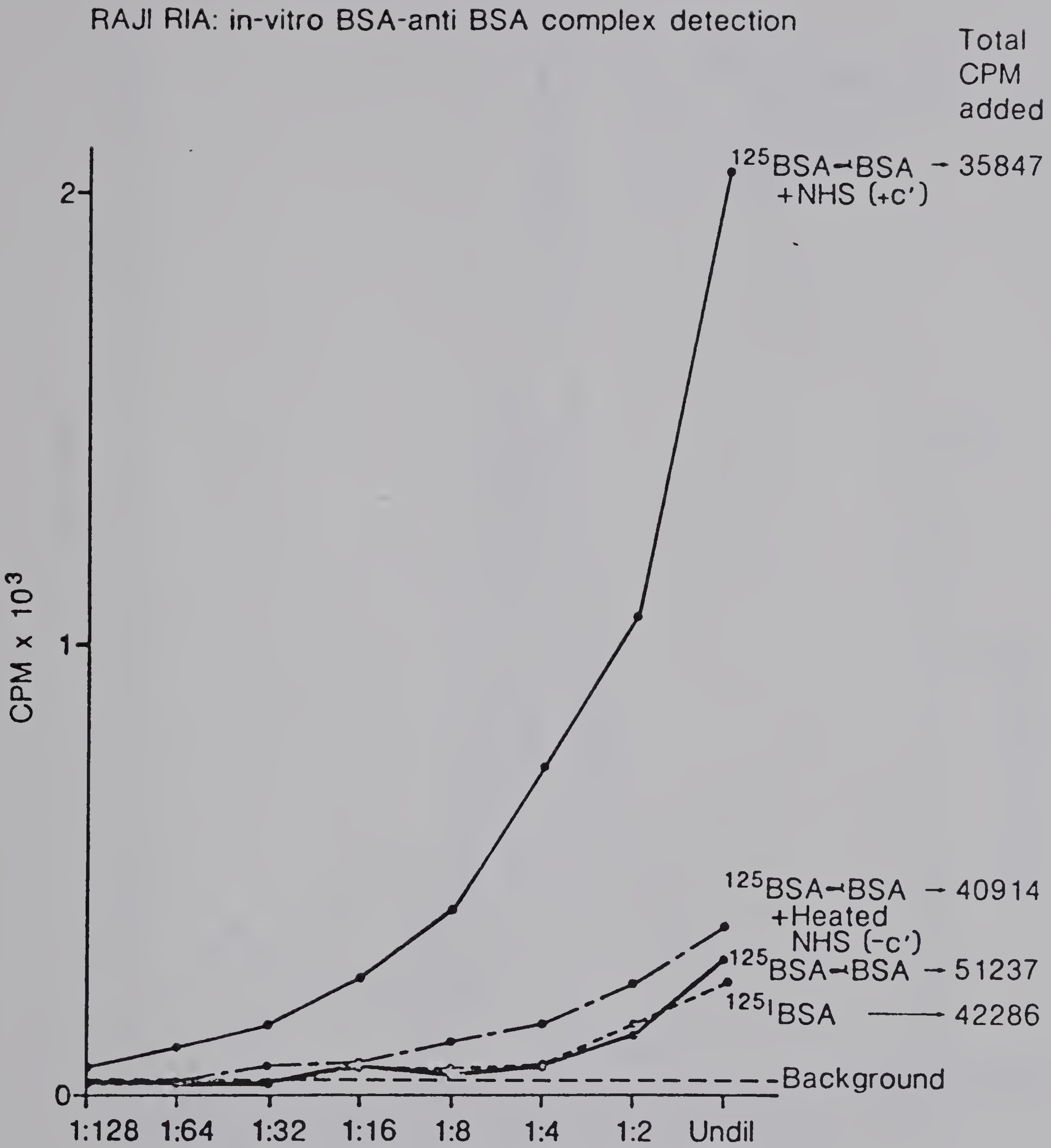

Fig. 7. Raji-RIA: in vitro BSA-anti-BSA complex detection.

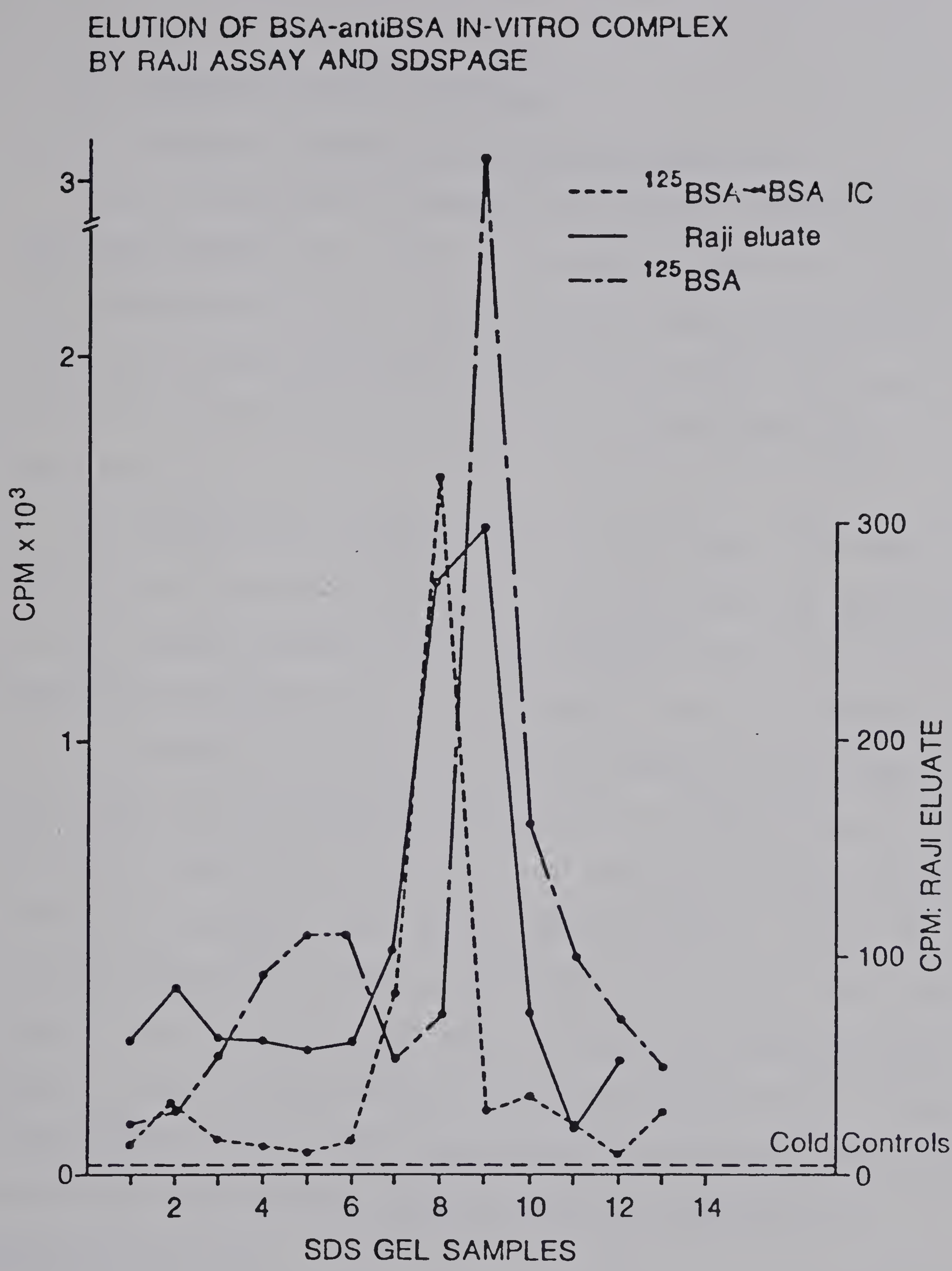

Fig. 8. Elution of BSA-anti-BSA in vitro complex by Raji assay and SDS-PAGE

5. Evaluation of False Positives

a) Influence of serum containing higher IgG levels

Raji cells have IgG-Fc receptors (FcR) besides having very avid complement receptors, eg. C3B, C3. It is possible that, at high serum IgG concentrations, Ig could bind to IgG-FcR on Raji cells and subsequently be picked up by the ^{125}I anti-human IgG used in the second step of the Raji-RIA assay. This could give rise to false positive assays for CIC.

We examined human sera containing monoclonal peaks ('M' peaks) of IgG and other immunoglobulin classes. Serum IgG levels, as quantitated by radial immunodiffusion, varied in these samples from normal to hyperglobulinemic range and over. Detailed results are given in Table 10.

At a normal range of serum IgG (5 to 20 mgm/ml) and with hyperglobulinemia of 20 to 40 mgm/ml, there was only slight evidence of false positivity in Raji-RIA, but this tendency increased with IgG concentrations above 40 mgm/ml. Sera with 'M' peaks of IgA, IgD, and IgM did not cause any Raji positive results, as expected. On the other hand, CIq-BA showed positivity at all ranges of IgG levels and by non-IgG bearing monoclonal sera. This is because ^{125}I CIq binds to any highly charged anionic proteins (6b, 30, 229) (eg. heparin) and PEG precipitation, used in this assay, also causes cryoprecipitation and aggregation of immunoglobulins (2).

b) Influence of serum containing antilymphocytic antibodies

Raji assay involves first absorption of C3 bound CIC onto C3b and C3d receptors on the Raji cell surface, allowing subsequent detection and quantitation by radiolabelled anti-human IgG. Raji is a B lymphoblastoid cell line established from a patient with Burkitt's lymphoma.

TABLE 10

Evaluation of False Positives in CIC Assays:

(a) Influence of Myeloma Sera[x] in Raji RIA and CIq-BA

Serum Containing 'M' Peaks	Number of Different Samples	CIC Positivity*	
		Raji RIA	CIq-BA (PEG)
A. IgG - 'M' peaks serum			
IgG levels (mg/ml)			
i) 5-20 - normal range	11	0	5
ii) 21-40 - hyper-globulinemic range	6	1	4
iii) > 40	3	2	2
Subtotal	20	3 (16.6%)	11 (55.0%)
B. IgM-'M'	4	0	2
C. IgA-'M'	1	0	1
D. IgD-'M'	1	0	1
Total	26	3	15

* Positive values = values 2 S.D. above the mean ($\bar{X}$) values of normal human sera (NHS).

x Identification and quantitation of 'M' peak containing sera was done by Dr. Salkie, by radial immunodiffusion and immunoelectrophoresis.

It bears HLA-A, B, C, DR and other determinants (49) and therefore might give false positive results for CIC test sera containing antibodies to such determinants.

Theofilopoulos et al. (199) demonstrated that cold-reacting IgM-type antilymphocytic antibodies in SLE serum would not interfere with Raji-RIA as incubation with test sera is done at 37^{o}C and not at 4^{o}C, and F(ab')^{2} fragments of IgG isolated from SLE sera bind poorly to Raji cells at 37^{o}C (197). Woodroffe et al. (226) report that 27% of systemic lupus erythematosus (SLE) patients' sera contain antilymphocytic antibodies which could affect interpretation of the Raji assay; they used Raji cells as targets in a complement dependent microcytoxicity assay using dye exclusion as the endpoint. The literature has no detailed evaluation of the interference by antilymphocytic antibodies on Raji-RIA other than in SLE even though this limitation is often cited (2, 9).

Our objective was to examine this problem of Raji assay with a new approach. In this study we have used the system of antibody dependent cellular cytotoxicity (ADCC) with ^{51}Cr labelled Raji cells as target, or ADCC(Raji). This system is complement independent; antibody coated Raji cells are lysed by human K cells, target lysis being measured by ^{51}Cr release. The K cell killing of Raji cells is mediated by warm-reacting IgG antibodies directed against determinants on Raji cell membranes. The system is ideal for detection of warm-reacting IgG antilymphocytic antibodies which might give false positive CIC detection by Raji-RIA.

Using ADCC(Raji) we have evaluated three groups of selected sera from (a) multiparous women, (b) renal transplant recipients and (c) patients with SLE. Results of ADCC(Raji) were to be compared with Raji-

RIA for CIC to note the incidence of false positive reactions which could be attributed to these antibodies.

Materials and Methods

Serum samples were collected from (a) 25 renal transplant recipients at various intervals after allografting, (b) 21 SLE patients at various levels of disease activity, and (c) 21 multiparous women's sera, known to contain HLA-A, B, C or DR antibodies after screening by well established technique (150, 190). All serum samples were aliquoted and stored at $-70^{o}C$ and thawed only once for the use in CIC assay. For ADCC(Raji), serum samples were heat inactivated at $56^{o}C$ in a water bath for 30 minutes.

ADCC(Raji) System

Target cells: 10×10^{6} Raji cells in 100 microlitres of RPMI media with 10% FCS were labelled with 100 microlitres of ^{51}Cr as sodium chromate (Amersham, Canada) at $37^{o}C$ for 45 minutes in 5% CO_2 atmosphere. Labelled Raji cells were washed 3 times and resuspended in RPMI media with 10% FCS at a concentration of 2×10^{6} per ml.

Effector cells (K cells): were prepared from heparinized human blood using Ficoll-isopaque gradient separation. Human peripheral blood lymphoctyes (PBL) of one donor (MDG) were used in all experiments to exclude any influence due to variation of effector cells. Final concentration of effector cells was 10×10^{6} per ml in RPMI media with 10% FCS.

Serum

Each test serum was used in quantities of 100 microlitres per test in serial dilutions to 1/16.

Serum STIN was used as a positive control in all ADCC(Raji) experiments. This serum was obtained from a multiparous woman and contains antibody to a public or 'broad' DR specificity which includes DR3 and 6, as described previously from this laboratory (94). It had been established that this serum achieved cell lysis by an ADCC mechanism.

Procedure for One-Step ADCC(Raji)

Tests were performed in 10 x 75 mm glass tubes with 100 microlitres of effector cells and 100 microlitres of test serum, in appropriate dilutions, in RPMI media with 25 microlitres of ^{51}Cr labelled Raji target cells. Final target to effector ratio was 1:20. Positive controls were set up for each experiment with serial dilutions of STIN sera; negative controls consisting of target and effector cell combinations with no serum added. After mixing, tubes were incubated at $37^{o}C$ in 5% CO_2 atmosphere for 4 hours; then 2 ml of cold normal saline solution were added and supernatants separated from cell pellets after centrifuging at $4^{o}C$ at 1500 rpm for 7 minutes.

Procedure for Two-Step ADCC(Raji)

In this method preparation of the effector cells, medium and other test conditions was the same as in the 1-step method except for the following:

i) Raji cell target preparation and pre-incubation with test serum: The method of target cell sensitization and labelling as described by Larsson, Perlmann and Natvig (99) for chicken red cells was adopted for Raji cells in the following manner: 3 x 10^6 Raji cells in 50 ml of medium were incubated with 100 ml of test serum and 100 ml of ^{51}Cr as sodium chromate (Amersham, Canada) at $37^{o}C$ for 45 minutes in 5% added CO atmosphere. At the end of the incubation period, cells were

washed three times in 50 times excess volume of medium and counts adjusted to 2×10^6 cells per ml.

ii) Effector cells were then added at an effector to target ratio of 20:1 and

iii) incubated for four hours at $37^{\circ}C$ in 5% added CO_2 atmosphere.

iv) The rest of the procedures were the same as those of the 1-step procedure described above. Each sample was run in duplicates with appropriate controls included in each experiment. STIN serum and other sera from hemodialysis and transplant patients were used as positive controls while sera from normal persons served as negative controls.

Chromium Release Calculation for Both ADCC Systems

^{51}-chromium release was calculated from:

$$^{51}\text{Cr release (\%)} = \frac{\text{cpm in supernatant}}{\text{cpm in supernatant + cpm in cell pellet}} \times 100$$

and percentage of specific ^{51}Cr release from:

specific ^{51}Cr release(%)

$$= \frac{\text{experimental } ^{51}\text{Cr release - background } ^{51}\text{Cr release}}{\text{maximum } ^{51}\text{Cr release - background } ^{51}\text{Cr release}} \times 100$$

Maximum ^{51}Cr release from ^{51}Cr labelled Raji cells was measured by adding 500 microlitres of hemolyte and was about 90%. Background release, as determined from eight consecutive experiments, varied from 11.5 to 34% with a mean of 20.11 ± 8.84 (S.D.). All tests were done in duplicate. Variations between duplicates were less than 3%. Mean values from the duplicates of each serum were employed for calculation of percent ^{51}Cr release and specific ^{51}Cr release (%). Any serum showing specific ^{51}Cr release greater

than 9.9 at 1:16 dilution was considered to be positive. Qualitative assessment of specific ^{51}Cr release observed at 1:16 dilution of any serum was designated as follows:

Specific ^{51}Cr release (%) of:

0 - 9.9 = negative

10 - 19.9 = +

20 - 29.9 = 2+

30 - 39.9 = 3+

40 - 49.9 = 4+

$\geq$ 50 = 5+

Complement Dependent Cytotoxicity (CDC) with ^{51}Cr Raji Cell Targets: CDC(Raji)

This was performed by the standard protocol in our laboratory for CDC(B) except that we have used ^{51}Cr labelled Raji cells as targets and determined specific chromium release instead of dye exclusion as an end point. Briefly, 25 μl of test serum were incubated with 25 μl ^{51}Cr labelled Raji target cells (1 x 10^6/ml) at 37^0C x 1 hour (same incubation temperature as for Raji RIA) and then 25 μl of low tox rabbit complement at an appropriate dilution were added, followed by a period of two hours' incubation at 37^0C (final incubation). Reaction was terminated by adding 2 ml of cold saline, centrifuged at 500 g for 7 minutes, and supernatant and pellet counts were measured in a gamma counter. Background reactions were obtained by not adding complement to the targets, total chromium release was obtained by counting after the addition of hemolyte solution. Tests were run with appropriate positive samples (sera from transplant and multiparous women) and negative

controls (normal human sera and AB serum) in each test. Specific chromium release was calculated by the same procedure as the ADCC system, cited above. Mean specific ^{51}Cr release (%) observed among six different normal individual sera and pooled normal human sera was 15.92 ± 5.0 (S.D.) and that of a strongly positive control serum (VE, a renal transplant rejected patient) was 88.5 ± 3.62 (six consecutive experiments). Therefore, specific chromium release greater than 20% in a test serum was considered to be positive and above 50% as strongly positive.

Determination of antibody class, in the test sera, was done by their sensitivity to dithiothreitol (DTT) as described by Pirofsky et al 1974 (156) and Roy et al 1981 (163). IgM antibodies are destroyed by this substance as opposed to IgG. Reduction of specific chromium release to 50% or less than the untreated serum sample was taken as DTT positive reaction.

Raji cell assay for CIC (Raji-RIA) - as described in page 16.

Results

^{51}Cr release from Raji target was determined in four consecutive experiments of ADCC(Raji) to establish consistency and reproducibility in the positively reacting STIN serum. Specific ^{51}Cr release (%) was 79.39 ± 2.75, 75.73 ± 1.48, and 70.65 ± 6.68 (S.D.) at 1:2, 1:4, and 1:16 dilutions, respectively. A typical ADCC(Raji) experiment is shown in Table 11. It is of interest to note that only the IgG fraction of serum STIN showed ADCC activity.

Results of ADCC(Raji) and Raji-RIA (for CIC) are compared in Tables 12, 13 and 14. Table 12 shows that only six of 25 trans-

plant sera were ADCC positive against Raji cells. Of these 3 were positive for CIC by Raji-RIA. Among 19 sera negative for ADCC, 8 were positive for CIC. Thus there was no correlation between antibodies directed towards Raji cell membrane antigens by ADCC(Raji) and CIC as detected by Raji-RIA, ($x^2 = 0.11$, $p > 0.95$) in these sera.

Results from multiparous women's sera are given in Table 13. In 21 such sera, 9 were positive by ADCC(Raji) of which 4 were also positive for CIC and for Raji-RIA. In 12 sera negative in ADCC(Raji), 3 were positive for CIC. These multiparous women's sera can be subdivided by whether or not their HLA antibodies, as determined by independent testing against panels of HLA-typed lymphocytes, were directed against HLA antigens known to be present on Raji cells. ADCC(Raji) positivity was noted to be higher in those sera with specificity against Raji HLA antigens, but this group showed lower incidence of CIC positivity by Raji-RIA. Some of the strongest reactors in ADCC were negative for CIC in Raji-RIA, e.g. STIN, G256B and X200B. There was no correlation between CIC and ADCC in this group of multiparous sera ($x^2 = 0.87$, $p > 0.75$).

In the SLE group 19 of 21 sera (90%) were positive for CIC, most of them quite strongly (Table 14), but only one was positive in ADCC(Raji). This unexpectedly low incidence of 1-step ADCC(Raji) positivity in SLE sera could possibly be due to blockade of K cell Fc receptors by immune complexes which would prevent detection of antibodies directed to Raji cell targets. To examine this possibility the author re-evaluated the SLE sera by the 2-

step ADCC(Raji) system. In this system Raji cell targets were pre-incubated with the test serum and washed three times. Effector cells were then added and final incubation at 37°C was carried out for four hours (see Methodology). Results of 2-step ADCC(Raji) among control sera are shown in Table 15 and those of SLE patients in Table 16. There are no significant differences between the 1-step and 2-step ADCC(Raji) results for SLE sera (see Table 16), indicating absence of warm reacting antibody activity against Raji cell targets and no evidence for inhibition of effector cell activity by presence of immune complexes. Additionally, we tested SLE sera for complement dependent reactivity against Raji cell targets by CDC(Raji) chromium release assay at 37°C. Four of the 21 SLE sera were positive by CDC(Raji) but only one was mediated by IgG antibody as determined by DTT reaction (see Table 16). In summary, it was found that only low reactivity by SLE sera against Raji cell targets at 37°C by either ADCC and CDC reactions and believe such reactivity is not an important cause of false positivity in CIC (Raji) assays.

Discussion

Raji cells have HLA-A3, A11, BW4, BW6 and DR3 and DR6 determinants on their surface (49). Multiparous women's sera selected because of CDC antibodies directed against these HLA and DR specificities, also had a high degree of reactivity in ADCC(Raji). ADCC(Raji) was negative in all but two sera containing no CDC antibody to Raji. In one of these the presumed DR specificity had not been defined by ADCC(panel cells); in the other serum, WEIRSMA, broad reactivity had previously been found in ADCC(panel

cells) to be directed against a public specificity on B lymphocytes by (Table 13, sera 17 and 20).

Anti HLA-antibodies frequently occur after multiple pregnancies or renal transplantation. Such sera could give false positive results for CIC by Raji-RIA. In these two groups of sera there was considerable reactivity in both Raji-RIA and ADCC(Raji) (Tables 12 and 13), but the poor correlation between these two tests indicates that ADCC antibodies against Raji cell markers are not the cause of false positive tests for CIC under the operational conditions of Raji-RIA.

In selected SLE sera, CIC by Raji-RIA were detected with high frequency. This is expected as most of the sera were from patients suffering from active disease. This group had the lowest incidence of ADCC antibodies directed against Raji markers. It was conjectured that one reason for this might be blockade of Fc receptors on K cells by CIC and thereby inhibition of ADCC reactions but this was shown not to be the case when SLE sera were then tested in the 2-step ADCC assay (Table 16) which involves pre-incubation with Raji cells with test sera, and then washing prior to addition of K cells (step 2).

Patients with SLE are known to have a high incidence of cold-reacting antilymphocytotoxic antibodies as detected by complement dependent assays (22, 125, 127, 173, 181, 191), but observations are discrepant regarding the optimal temperature for incubation. Some found maximum sensitivity of the CDC test at room temperature (181, 191) and others at colder temperatures (22, 191). Warm-reacting IgG antilymphocytic antibodies in SLE have been described

but seem to be present in only low amounts and may have only low affinity (221).

Woodroffe et al. (226), using SLE sera which were CIC positive in Raji assay, showed that 27% were positive for antilymphocytic antibodies by complement dependent microcytotoxicity against Raji cell targets, but performed at 20^{o}C. This different incubation temperature for CDC between their data and ours may be the explanation of discrepancy. Also, microcytotoxicity may be mediated by IgM whereas ADCC is mediated by IgG. Also, autocytoantibodies in SLE may be unable to bind to Raji cells in the conditions of the Raji-RIA assay.

CDC experiments were carried out with ^{51}Cr Raji targets at 37^{o}C showed only 1 out of 21 SLE sera to contain IgG against Raji cells. Oztuck and Terasaki (148) recently pointed out that in SLE, as in many other diseases, not only is antilymphocytotoxic activity (as determined by CDC) predominantly present at a colder temperature (5^{o}C) and is mostly directed against non-DR B cell determinants, but these reactions became very weak at 37^{o}C. They postulate that these non-specific cold-reacting lymphocytotoxins are directed against surface Ig of B lymphocytes and form complexes which are shed off at 37^{o}C. Raji cells lack in surface Ig (192-194); this may be another reason for being unable to correlate cold (5^{o}C) or warm (20^{o}C) lymphotoxins (against PBL or B panel cells) with possible false positivity in Raji-RIA. Anderson et al (9) have recently described increased incidence of warm reacting antibodies to Raji cells in Raji RIA positive SLE sera. They used pronase digestion to strip off Raji cell complement

receptors and compared ^{125}I goat-anti-human Fcγ uptake of pronase digested with non-digested Raji cells. SLE sera, positive by Raji-RIA, the difference was found to be minimal suggesting this is due to presence of anti-Raji antibodies causing false positivity for CIC. The authors could not exclude the possibility that remaining Fc receptors (FcR) were still participating in the pronase digested Raji cells, by carrying out appropriate blocking experiments. Pronase digestion can also induce membrane alterations but ADCC reaction used by us avoids such alterations of Raji cell membrane.

ADCC reactivity could be enhanced by immune complexes of certain characteristic lattice structures (229) but that did not seem to be the case amongst the sera of our SLE patients as ADCC(Raji) by either 1 and 2 step reactions was the same. Although SLE sera appear not to have warm reacting antibodies to Raji cells (and low false positivity in Raji-RIA for CIC) such antibodies are present in the sera from multiparous women and after transplant rejections. These are probably HLA related, are easily detected by the ADCC(Raji) reaction, but even so showed no statistical correlation with results by Raji RIA for CIC.

Conclusion

It is concluded that IgG antibodies to Raji membranes are not a significant cause of false positivity results in CIC detection by Raji RIA.

TABLE 11
Results of ADCC(Raji)* Experiment

Serum Samples	Dilutions	^{51}Cr Specific Release (%)	Qualitative Positivity ø (1 - 5+)
STIN serum	1:2	85.74	
	1:4	79.07	5+
	1:16	84.22	
** IgG Fraction of	1:40	50.69	
STIN Serum	1:160	54.17	5+
	1:640	52.68	
** IgM Fraction of	1:30	2.53	
STIN Serum	1:120	5.34	-
	1:480	5.84	
DN: (Transplant patient)	1:2	12.61	
Serum 24, Table 2	1:4	6.40	-
	1:16	2.77	
BH: (Transplant patient)	1:2	73.56	
Serum 25, Table 2	1:4	71.01	+
	1:16	16.52	
FD: (SLE patient)	1:2	3.95	
Serum 21, Table 4	1:4	9.53	-
	1:16	0.94	
HG: (SLE patient)	1:2	20.19	
Serum 18, Table 4	1:4	23.87	+
	1:16	19.39	
9117A (Multiparous female)	1:2	-3.68	
Serum 21, Table 3	1:4	-1.46	-
	1:16	5.33	
X733 (Multiparous female)	1:2	50.69	
Serum 3, Table 3	1:4	54.13	5+
	1:16	53.09	

* Target = ^{51}Cr labelled Raji cells, Effector cells - human PBL cells from one donor, E:T ratio = 20:1, Final incubation 4 hours at 37^{o}C in 5% CO_2 atmosphere.

** IgG and IgM fractions were obtained by gel filtration of STIN sera in a Biogel A.5m column.

ø Qualitative positivity: assessed from specific ^{51}Cr released (%) observed at 1:16 dilution

0 - 9.9 = - negative
10 - 19.9 = +
20 - 29.9 = 2+
30 - 39.9 = 3+
40 - 49.9 = 4+
$\geq$ 50 = 5+

TABLE 12
ADCC(Raji) and Raji-RIA Results: Transplant Patients

Serum	Raji RIA**	ADCC(Raji)*
HL	10.8	43.67 (4+)
ME	7.0	1.78
LW	6.0	-1.75
CD	4.6	5.65
DN	4.0	2.77
MK	3.9	-1.98
DE	3.7	40.26 (4+)
RB	3.2	33.11 (3+)
SA	2.4	-0.23
LD	2.3	-2.35
SB	2.0	-0.25
GE	-	-5.04
MA	-	0.15
WR	-	0.42
KY	-	-0.81
HJ	-	2.06
BT	-	9.03
GU	-	12.79 (+)
GS	-	32.10 (3+)
SS	-	0.23
WE	-	5.90
RD	-	-3.87
JI	-	-2.13
CL	-	3.31
BH	-	16.52 (+)

* See footnotes * and ø of Table 11, expressed as % specific ^{51}Cr release

** Positive results above 2 S.D. of normal controls
- = negative (< 2 S.D. of normal controls).

TABLE 13
ADCC(Raji) and Raji-RIA Results: Multiparous Females

Serum	HLA Antibodies ø (where known)	Raji RIA** (≥ 2 S.D.)	ADCC(Raji)*
Group I: Sera with antibodies to Raji HLA antigens ∞			
X733		6.8	53.09 (5+)
Martha D.	DR3	4.9	33.93 (3+)
X929		2.1	1.62
X898		2.0	29.76 (2+)
X731		-	-4.42
X732		-	-0.34
X734	Multi	-	4.23
X752	HLA	-	-5.12
X759	A, B, and C	-	6.02
X899		-	4.79
8768B	DR6, B5	-	6.51
X200B	A3	-	51.40 (5+)
G256B	Broad Anti-B	-	53.72 (5+)
STIN	DR3, DR6 ø	-	66.73 (5+)
Group II: Sera with antibodies to HLA determinants not present on Raji cells			
9117A	A2	11.9	5.33
9444B	B12	3.5	-0.03
4000G	A1, DR	2.1	54.34 (5+)
7206B	A1, B12, DR4	-	1.82
8032B	DR7	-	8.24
92102B	B5, BW35	-	10.63 (+)
Weirsma	DR4	-	18.69 (+)

* See footnotes * and ø of Table11, expessed as % specific ^{51}Cr release

ø Detected by complement dependent cytotoxicity assay CDC(B) except serum 14 (STIN) which is done by ADCC(B), Ref 14

∞ Raji HLA/DR antigens: A3, A11, BW4, BW6, DR3, DR6, Ref 15

** Positive results above 2 S.D. of normal controls

- = negative (< 2 S.D. of normal controls).

TABLE 14
ADCC(Raji) and Raji-RIA Results:
Systemic Lupus Erythematosus (SLE) Patients

Serum	Disease∞ Activity	Raji-RIA* ($\geq$ 2 S.D.)	ADCC(Raji)**
JL	A	39.0	0.29
PK	A	20.5	2.17
ZA	A	19.2	1.58
SL	A	18.9	4.36
LJ	A	17.4	1.16
SS	A	16.9	4.93
EH	A	16.8	-0.90
MA	A	16.2	0.97
AL	A	15.9	-4.41
MWK	A	15.5	-1.02
KR	A	15.3	1.57
RY	A	14.6	0.42
FD	A	13.3	0.94
MM	A	11.5	1.06
SE	A	10.7	-0.32
EA	A	10.4	-2.75
HG	A	4.5	19.39 (+)
SM	A	3.2	-1.41
SB	A	2.6	3.39
FJ	IA	-	2.94
FL	IA	-	-2.94

* See footnotes * and ø of Table11, expressed as % specific ^{51}Cr release

∞ A = active, IA = inactive

TABLE 15
ADCC(Raji): Comparative Results of 1-Step and 2-Step Tests

A. Controls
ADCC (RAJI)

	1 - STEP		2 - STEP	
Serum	Sp $^{51}Cr.R(\%)$	Score +	Sp $^{51}Cr.R(\%)$	Score +
NHS 1	6.1	-	5.0	-
	6.8	-	3.9	-
	6.9	-	1.3	-
NHS 2	8.2	-	5.5	-
STIN (MP ♀)	71.9	5+	32.8	3+
	70.7	5+	20.9	3+
	77.5	5+	22.4	3+
Sera of Renal Transplant and Hemodialysis Patients **				
VE	73.0	5+	61.5	5+
	84.8	5+	66.8	5+
	76.9	5+	54.7	5+
BR	84.1	5+	42.8	4+
LA	80.7	5+	53.2	5+
DR	75.4	5+	57.3	5+
SE	47.3	4+	-2.5	-
ND	42.9	4+	13.3	+
CGE	39.3	3+	3.1	-
BS	7.9	-	0.5	-

* STIN = CDC(B) Negative

** ALL SERA CDC(B) Positive to panel cells (variably)

\+ See footnotes ** and ɸ of Table 11

TABLE 16
B. ADCC and CDC (Raji) Results in SLE Patients

	Specific ^{51}Cr Release (%)			
	CDC (RAJI) ‡		ADCC (RAJI) *	
Serum		DTT ° Rx	1 STEP	2 STEP
ZA	90.16 +	51.22	1.58	0.97
MA	88.15 +	41.00	0.97	4.22
JL	82.11 +	48.20	0.29	3.76
MM	47.72 +	53.03	1.06	1.62
SM	19.07		-1.41	5.54
FD	15.70		0.94	4.06
FL	15.18		-2.94	1.62
RY	14.46		0.42	2.24
SL	12.81		4.36	ND
FJ	12.69		2.94	3.99
SS	12.65		4.93	4.39
PK	11.55		2.17	7.32
AL	11.45		-4.41	2.84
SB	11.28		3.39	3.19
KR	9.72		1.57	2.87
HG	8.10		19.39 (+)	0.95
MWK	6.50		-1.02	1.75
EH	6.50		-0.90	4.32
LJ	6.12		1.16	3.21
EA	5.50		-2.75	3.54
SE	4.52		-0.32	4.42

* See footnote of Table 11.

‡ CDC (Raji): Complement dependent cytotoxicity tests carried out with ^{51}Cr labelled Raji cell targets at 37°C with low toxicity rabbit complement (see Methodology). Specific ^{51}Cr release (%) greater than 20 is considered a positive test.

° DTT - Dithiothretiol - 0.01M solution, pre-incubated with test serum at 37°C for 15 minutes; abolishes IgM bearing reactions (Ref 163). Reduction of specific ^{51}Cr release of 50% or higher than untreated sample is considered to be a positive test.

Chapter IV: Cystic Fibrosis and CIC

1. Review of Literature

Cystic fibrosis (CF) is a genetic disease and one of the commoner inherited diseases amongst Caucasian children occurring with a frequency of 1 in 2000 births. Disease is transmitted as an autosomal recessive trait and has no correlation with HLA antigens (58, 98). Clinically the disease is characterized by abnormally thick secretions from mucus glands which lead to pancreatic insufficiency or pulmonary insufficiency. There is also increased concentrations of sodium and chloride in sweat.

The main clinical features are related to pancreatic insufficiency and chronic, diffuse, obstructive pulmonary disease.

Early diagnosis, prophylactic therapy and frequent laboratory clinical examinations have resulted in a better life expectancy in these patients. It has improved from 2 years in 1948 to 19 years at present (32). Despite progress in overall management of these patients recurrent pulmonary infection still remains the major cause of death among this group of patients (225).

2. Lung Lesions and Immunity in CF Patients

CF children are born with normal lung structure (15, 46, 225) and protective function, eg. intact bronchociliary propulsive function, and alveolar macrophage phagocytic function (15, 17, 19, 46, 225) (non-specific protection). There also is normal specific immunologic protection from serum and bronchial secretions containing normal concentrations and functions of immunoglobulins (178, 187), complement proteins (169, 213) enzymes and B and T lymphocytes (73, 75).

The earliest pulmonary lesions are dilated and hypertrophied bronchial glands with metaplasia of goblet cells of bronchiolar epithelium (15, 46, 232). Thick viscous secretions from these hypertrophied glands lead to plugging of peripheral airways and functional obstructive lung disease initially. Later infection superimposes purulent secretions leading to bronchiolitis, bronchitis and bronchiectasis.

Gradually, repeated infections cause permanent lung damage and obstructive bronchopulmonary disease. Autopsy findings from 82 CF patients did not reveal emphysema below the age of 2 years but this finding increases progressively in older patients (15). Obstruction by chronic infection and more tissue damage causes a vicious cycle, with progressive loss of pulmonary functions and eventually death; this is the major cause of death in patients with CF (42, 46, 72).

Lung lesions are not specifically related to any specific infecting micro-organism but certain pathogenic peculiarities have been noted. The initial flora usually includes staphylococcus aureus which is then replaced by gram-negative bacteria such as Pseudomonas aeuroginosa (PA); the latter has become the predominant inhabitant in recent years (71, 123), indicating a change of flora related to antibiotic therapy. Smooth strains of PA are soon replaced by "mucoid" strains; this is rarely encountered in any other form of pseudomonas infection (46, 71, 114). However, pseudomonas vasculitis (which is characteristic of PA bacteremia) has not been observed in CF patients (50, 51). Other micro-organisms like Haemophilus influenzae, Proteus, E. coli, and Aspegillus have also been found in these patients with a low prevalence, and systemic infections from these micro-organisms are rarely ever reported in

CF patients. The infection is localized to intrabroncho-alveolar lung tissue only (17, 225).

Humoral immune responsiveness to bacterial infection remains intact. CF patients produce greater levels of antibody to PA, staphylococcal and Haemophilus infection than other patients with infection from the same agents (46, 71, 72, 74). No defect has been found in humoral response to routine immunization in CF patients using various bacterial polysaccharide antigens and measles, poliomyelitis and influenza vaccines (32, 72). Lipopolysaccharide antigens of Pseudomonas (PA) also provokes normal humoral responses even in CF patients who already have Pseudomonas infection (72, 153). Nor is there evidence of defective function of neutrophils, complement proteins, B and T lymphocytes or alveolar macrophages although there is early loss of propulsive bronchociliary transport mechanisms (non-immunologic) (46).

Because of these observations (of intact immune responsiveness to chronic persistent pulmonary infection in CF patients) some have postulated that systemic immune complex mediated lung injury (Type III, Gell and Coombs) could occur in these patients in addition to the already ongoing progressive lung damage. Soluble bacterial antigens forming immune complexes (CIC) with specific antibodies would, according to this hypothesis, mediate immune-complex-mediating injury to the lungs (120, 136, 169).

The first report (120) favoring this concept was an autopsy study where immune complex deposits containing immunoglobulin, C_3 and C_5, were demonstrated by immunofluorescence in lung, trachea, stomach and pancreas. Immune complexes were then also detected in serum and sputum of living CF children. The antibody class of Ig in these complexes was

mainly IgG or IgM. After elution the Ig showed cross reactivity to various human tissue antigens, bovine serum albumin (BSA) and hemolysin.

A second report (169) showed evidence of immune complexes in serum, sputum and at dermal-epidermal junctions; these were more prominent in CF patients with PA infection than without it.

Thereafter other investigators found an increased prevalence of CIC CF patients compared to various control populations (16, 131, 136). A summary of these different observations is given in Table 17.

Attempts to characterize the antigenic moiety in CIC eluates have had different success rates in different laboratories. Some detected antigenic BSA or alpha-staphylococcus hemolysin (120) while others found antigens of PA (136) or lipopolysaccharides (LPS) of PA antigens in PEG precipitated IC (16). These studies are not conclusive, but point to the heterogeneous nature of sero-antigens.

Recently attempts have been made to use CIC results in interpretation of clinical course and monitoring of the need for antibiotic treatment in these patients (131, 132); this is similar to procedures recommended for patients suffering from subacute bacterial endocarditis (SBE) (13, 14, 122).

3. <u>Existing Problems in CF Patients and Role of CIC</u>

Recurrent and chronic lung infection, particularly with PA, remains the most frequent cause of death in CF patients (46, 71) and poses a very difficult therapeutic problem requiring early diagnosis. It has been shown that early diagnosis and aggressive therapy of PA infection reduces mortality in CF patients (71). Bacterial culture results are not always helpful in this regard and it is postulated that rising

TABLE 17
Immune Complexes (IC) in Cystic Fibrosis (CF) Patients

Authors	Immune Complexes Methods of Detection	Materials	CF Patients n	CF Patients % +ve	Controls n	Controls % +ve	Remarks
1. McFarlane et al (1975)	a) Immunofluorescence	a) Autopsy materials	2	Both +ve	1	-	a) Linear deposits of IgG, IgM, C3, C5 in trachea, lungs, stomach and pancreas
	b) Sephadex gel filtration and counter immuno-electrophoresis	b) sputum	6	all +ve	none	-	b) Ab class: IgG, IgA, IgM - heteroreactive
		c) serum	3	all +ve	none	-	
2. Schiotz et al (1977)	a) Immunofluorescence	a) dermal epidermal junction	21	81	-	-	a) Dermal-epidermal junction, deposits of IgM, Clq, C3, and fibrinogen
	b) Complement consumption test (CTT)	Serum	21				b) +PA patients has >12 preciipitation antibodies against complex PA antigens
		+PA infection	11	6+	None	-	
		-PA infection	10	1+	None		c) +PA patients has IgM tissue nonspecific antinuclear antibodies

Continued . . . .

3. Nielsen et al (1978)	CCT and rheumatoid factor binding assay (RFBA)	sputum	24				CIC in +PA vs-PA statistically sig ($P < 0.10$)
		+PA infection	12	11+	None	-	
		-PA infection	12	1+	None	-	
4. Moss and Lewiston (1980)	Clq-BA (PEG)	Serum	51	51	23 other resp. dis.	7.4	a)91% CF patients colonized with mucoid PA has serum precipitins against PA b)PA CIC in precipitin +ve vs PA precipitin -ve not statistically significant c)CIC has no correlation with PFT, serum Ig or C1 levels
5. Berdischewsky et al (1980)	IgG quantitation by RID in 4% PEG precipitates	Serum	20 IH pts	90	-	-	PA-LPS antibody and endotoxin activity in PEG precipitates

\+ patients, not %
+PA = pseudomonas infection
-PA = no pseudomonas infection
PFT = pulmonary function tests

anti-PA antibodies or rising levels of CIC might have greater prognostic value.

Studies have been made to see if antibody levels to PA antigen(s) clarify the question of actual reinfection versus mucoid colonization (228). Antibodies to PA are detected by various methods (hemagglutination, agar-gel precipitation, agglutination, etc.) (44, 63, 228). These assays have variable degrees of sensitivity and varying ability to distinguish between mucoid colonization and tissue re-infection. Crossed counter immunoelectrophoresis with PA complex antigens (71) and solid phase assay with PA-LPS antigens (131) are sophisticated techniques which have been described, but are also not completely free from cross reactivity with other bacterial antigens (71) and are not available on a routine basis.

Detection of IC in serum or sputum of CF patients is also under investigation to improve the diagnosis of tissue reinfection from mucoid colonization. Monitoring of CIC levels has been found useful in chronic (low grade) heart valve infection in subacute bacterial endocarditis (SBE). In this disease blood culture occasionally is negative yet disease activity correlates to CIC levels and has been successfully managed by antibiotic therapy dictated by changes in CIC levels (12-14, 122). Use of CIC levels in CF patients with infection would thus be of great clinical help in the antibiotic management of such patients if the evidence showed a similar correlation.

4. <u>CIC in CF Patients</u>

<u>Objectives of the study</u>

Our studies have been directed at:

a) Re-examination of prevalence of CIC in a CF population using

the modified Raji-RIA (not previously reported in the medical literature) in comparison to a method previously reported by others, the Clq binding assay, Clq-BA. Raji-RIA readily picks up IgG containing CIC which also bear activated complement components (C_3b and C_3d) and could be a better indictor of disease activity (194).

b) Examination of the relationship of important clinical parameters of CF patients and CIC levels:

(i) Pulmonary function tests

(ii) Significant bacterial growth in sputum (colonization counts)

(iii) Overall disease activity evaluated by the Shwachman scoring system.

c) Serial observations of CIC levels in some of the CF patients to note any characteristic patterns.

d) On the basis of above observations, to evaluate the value of CIC measurement in clinical management of CF patients

5. Patients and Methods

Patients

48 children attending the CF clinic of the University of Alberta Hospital, constituted the patient population. Median age of these patients was 10 years, ranging from 1-25 years. Twenty of 48 patients were female.

Clinical diagnosis and follow-up of these patients was done by Dr. F. Harley. No patient presented with any clinical and laboratory features suggestive of overt gastrointestinal disorders: Lung disease, with or without infection, was the main clinical presentation. Some

patients needed frequent hospitalization for control of pulmonary infections. Six patients died subsequently during the study period (three of them being females).

Serum samples for CIC were collected from each of these 48 patients on at least one occasion. Serial samples were collected from 30 of these patients, particularly those requiring medical checkups for chest problems. These constitute a specially selected sub-population of the CF population.

Sputum samples were obtained simultaneously for bacteriological culture as well as at more frequent intervals. In 29 patients pulmonary function tests were also measured on the same day as serum samples were collected for CIC. Laboratory tests were analyzed without knowledge of the simultaneous evaluation of clinical status.

Control groups:

a) Healthy children: Serum samples from 40 healthy children were collected concurrently. They formed a group of normal controls. Median age of this group was 9 years (range 2-25 years) and 24 of them were females.

b) Subacute bacterial endocarditis (SBE): 47 serum samples were tested for CIC from 14 patients (age 4-79 years) with confirmed diagnosis of SBE.

Methods:

a) CIC levels were measured by Raji-RIA and Clq-BA.

b) Sputum cultures: Quantitative bacteriology was done on specimens for full identification and antibiotic sensitivity on all usual pathogens such as Pseudomonas, Haemophilus, Staphylococcus, Group A Streptococcus.

Organisms isolated at a specific dilution were reported according to the following grading system:

<u>Grade</u>

0 = No growth

1 = Scant when it is cultured on only the undiluted plate.

2 = Few when it is cultured up to 10^{-2} dilution.

3 = Moderate when it is cultured up to 10^{-4} dilutions.

4 = Many when it is cultured up to 10^{-6} dilutions.

Growth of micro-organisms at grade 3 and 4 was taken as "significantly positive bacterial cultures".

c) Pulmonary function tests: Pulmonary function was determined by measuring vital capacity (VC) and forced expiratory volume in one second (FEV_1). These measurements were made using a Godalt Pulmotest. Predicted normal values for VC and FEV_1 were obtained from Weng and Levinson (214) and the CF data expressed as percent of predicted normal value. The sum of the percent of predicted values for VC and FEV, was used to obtain a pulmonary function score; the greater the score the better is pulmonary function (188). Analysis of other routine pulmonary function tests like residual volume (RV) and total lung capacity (TLC) were done but not analyzed, because they are known to have poor reproducibility in CF children (188).

d) Overall clinical severity of the disease activity of CF patients: This was determined by the Shwachman scoring system (Appendix 4). Scoring of individual patients was done independently by Dr. F. Harley who had no knowledge of the results of CIC, sputum cultures, or pulmonary function tests. The score was done on the same day as that on which specimens were obtained.

e) Stastical analysis between CIC levels and other parameters was done by Student's "t" test, Chi square, and analysis of correlation coeffecients with linear regression.

Results

Prevalence of CIC in CF patients was found to be significantly higher than in the healthy children of similar age group (Figure 9 and Table 18). This fact was born out from both single point and serial study results. Raji-RIA was more frequently positive than Clq-BA (Table 18) indicating, presumably, a higher prevalence of IgG complexes bearing fixed components of C_3.

The relationship of CIC prevalence and pulmonary functions is shown in Figure 10. Patients with CIC in serum have more deterioration of pulmonary function than those without ($p < 0.05$, Fig. 10). Also there is a highly significant correlation between the Shwachman score and the pulmonary function score ($p < 0.005$).

Significant degrees of bacterial growth (grade 3 and 4, positive at 10^{-4} to 10^{-6} dilutions) in CF sputum was also positively correlated with the presence of CIC in serum ($p < 0.01$). (81%) of significant bacterial growth was due to Pseudomonas aeuroginosa. Sputum culture results and Shwachman score also correlated significantly ($x^2 = 8.94$, $p < 0.005$).

6. Discussion

The correlation of CIC prevalence (in serum) with poor pulmonary function and significant degree of sputum bacterial growth (mostly with PA) suggests that the presence of CIC in serum would be associated with poor overall prognosis. This is further supported by the following:

Table 18

Prevalence of CIC Among Cystic Fibrosis (CF)

and Other Groups of Populations

Groups	CIC Positive Results			
	n	Raji RIA	C1q-BA	Combined
Normal controls 40 subjects	40	2 (5.0%)	1 (2.5%)	3 (7.5%)
CF patients (a) 48 patients single point study	48	18 (37.5%)	5 (10.4%)	20 (41.6%)
CF patients (b) 48 patients providing 175 samples (only 18 had one sample only)	173	63 (36.4%)	22 (12.7%)	74 (42.8%)
Subacute bacterial endocarditis 14 patients	45	31 (68.9%)	16 (35.6%)	36 (80.0%)

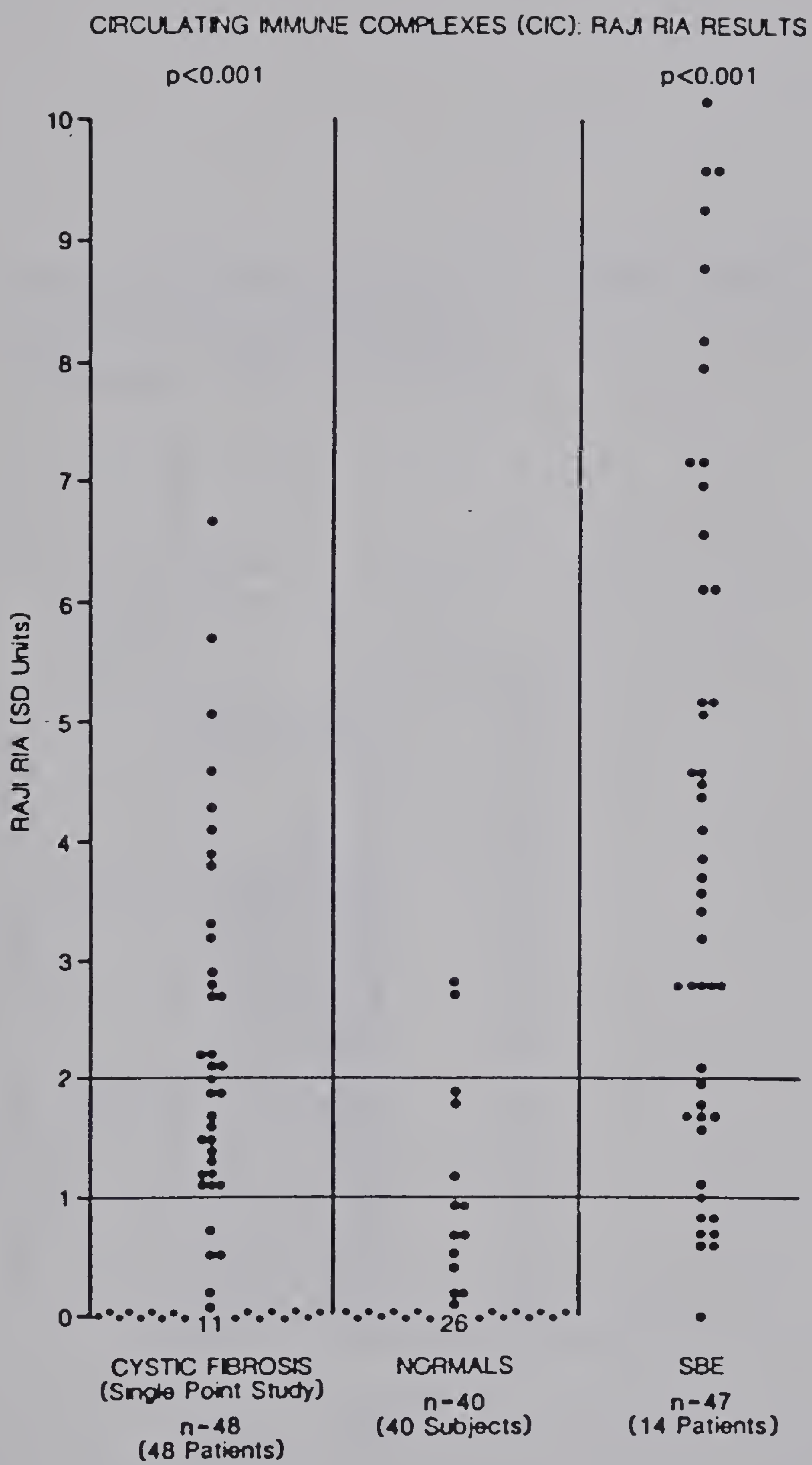

Fig. 9. Prevalence of CIC, as detected by Raji-RIA among cystic fibrosis patients and controls.

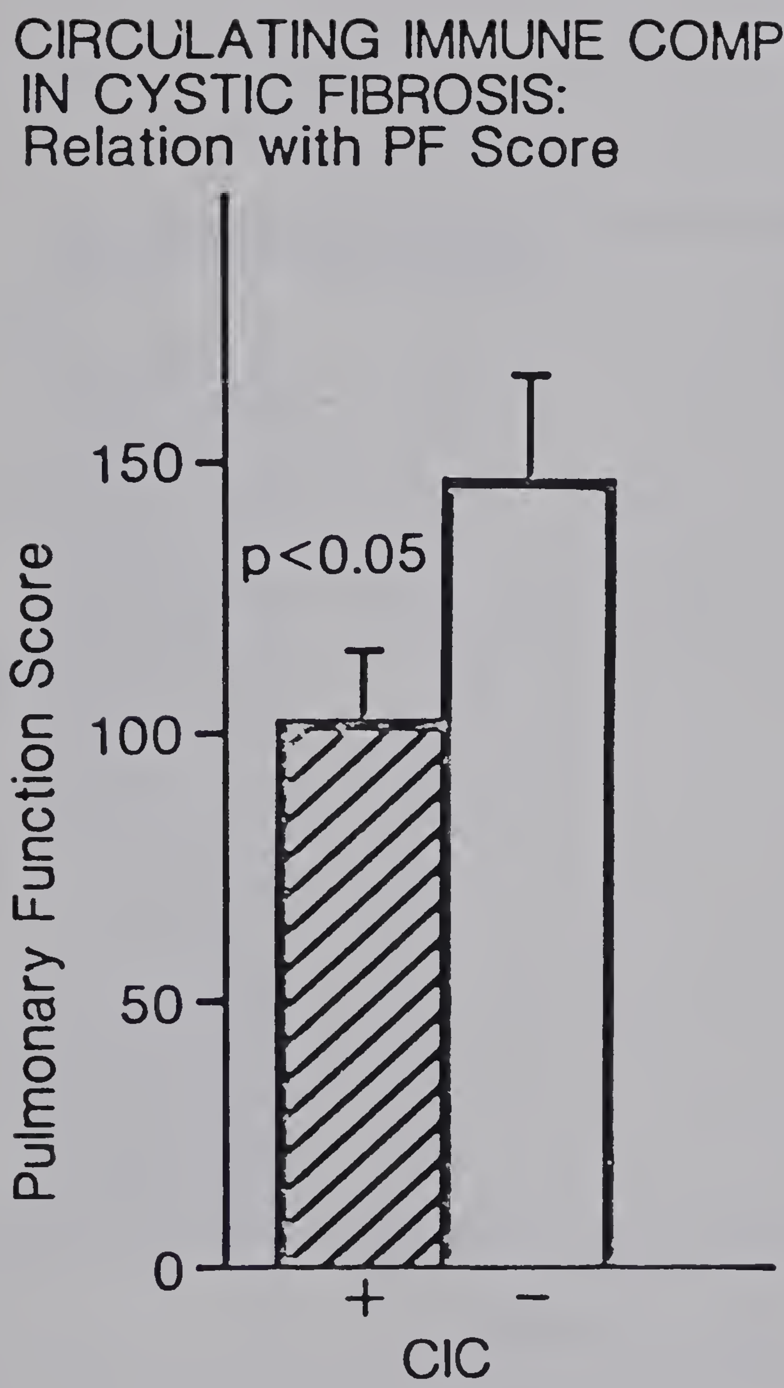

Fig. 10. Circulating Immune Complexes (CIC) in Cystic Fibrosis: Relation with PF Score

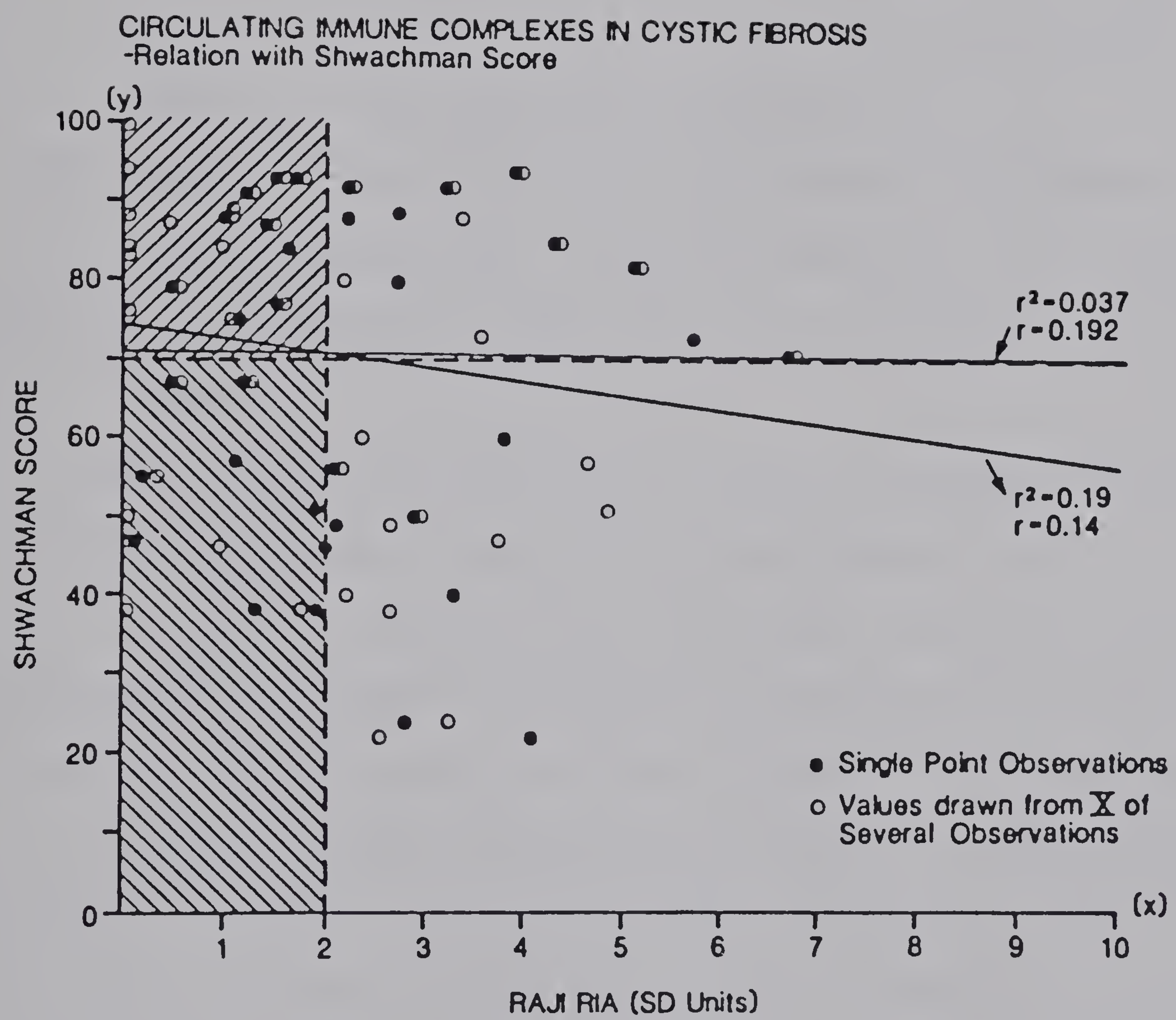

Fig. 11. Circulating Immune Complexes in Cystic Fibrosis: Relation with Shwachman Score

a) Six patients who died during the 10 months of this study all had CIC in their sera as well as recurrent pulmonary infections with Pseudomonas.

b) Overall severity of disease in CF is represented by the Shwachman scores. In this study, this correlates significantly with reduced pulmonary function scores ($p < 0.001$) and with significant sputum bacterial growth ($p < 0.05$). Prevalence of serum CIC correlates well with both PF scores and bacterial growth ($p = < 0.05$ and < 0.01, respectively).

This implies that CIC prevalence would correlate with disease severity or low Shwachman scores. However, examination of this point reveals poor correlation ($p > 0.05$) between CIC and Shwachman scores as shown in Figure 11.

A Shwachman score of 70 or below is evidence of advanced disease with permanent emphysema. Scores below 55 indicate more severe permanent lung damage. Analysis between a Shwachman score above or below 70 and prevalence of serum CIC shows that the serum of half of the more ill patients contain CIC, but half do not (Figure 11). This is equally true if the cutoff for the Shwachman score is 60 or 50, suggesting that CIC positive serum occurs only in about half of CF patients even when far advanced in disease (an observation somewhat in contrast to that reported in a) above).

Conclusions

Tentatively it is concluded that either:

a) Occurrence of CIC in CF patients may be an epiphenomenon and has no specific significance; or

b) CIC in serum of half the patients who have advanced to serious clinical stages is because there are two different mechanisms of advanced disease: one mediated by IC and the other non-IC mediated. Further, one would postulate that half of the advanced CF patients have a non-IC mediated process whereas the others may have both.

There is no conclusive evidence in the literature in support of this hypothesis other than suggestive findings as shown in Table 17 In attempting to find evidence to interpret these possibilities we made the following observations:

a) Serial observations in two identical twins having similar degrees of illness as measured by Shwachman scores at the starting point of the observations, showing two different patterns of prevalence of CIC (Fig 12). This does not indicate two forms of the disease and the reason for the striking difference is not apparent to us.

b) Other CF patients with comparable severity of clinical disease (measured by Shwachman score) and living in the same environmental conditions (brothers) also may have a similar dichotomy in CIC prevalence as seen in Figure 13.

c) Other patients with equal severity of illness can also show this disparity in prevalence of CIC as seen in Figures 14 and 15.

Secondly, the relationship of FEV_1/VC scores with CIC results was examined, it was noted that CIC negative patients had scores falling absolutely in the normal zone of lung functions (Figure 16), whereas others were mostly in obstrictive and obstructive (emphysema), and

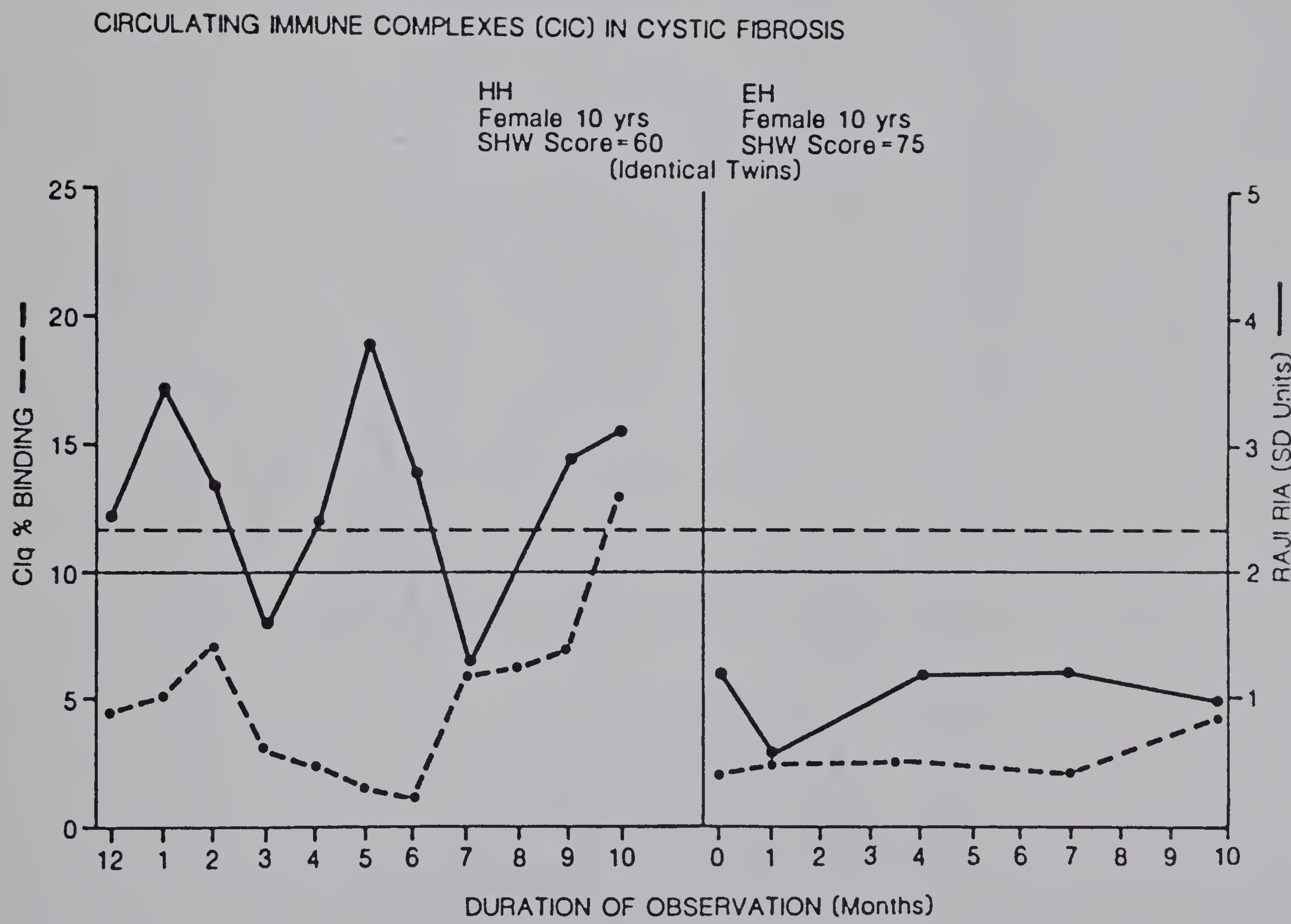

Fig. 12. CIC in Cystic Fibrosis, Longitudinal Studies by Raji-RIA and Clq-BA. Results in HH and EH, Identical Twins.

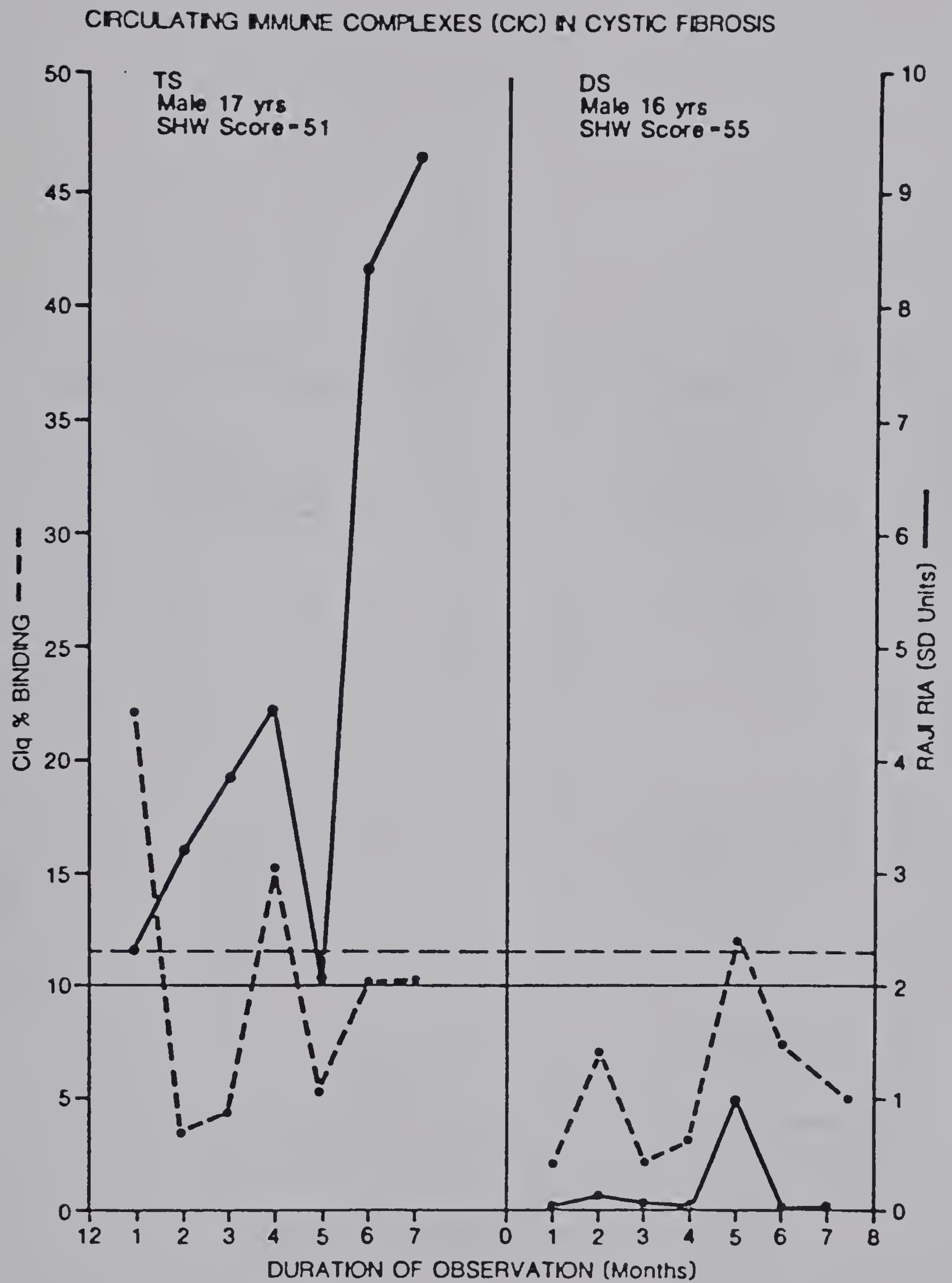

Fig. 13. CIC in Cystic Fibrosis, Longitudinal Studies by Raji-RIA and Clq-BA. Results in TS and DS, Brothers.

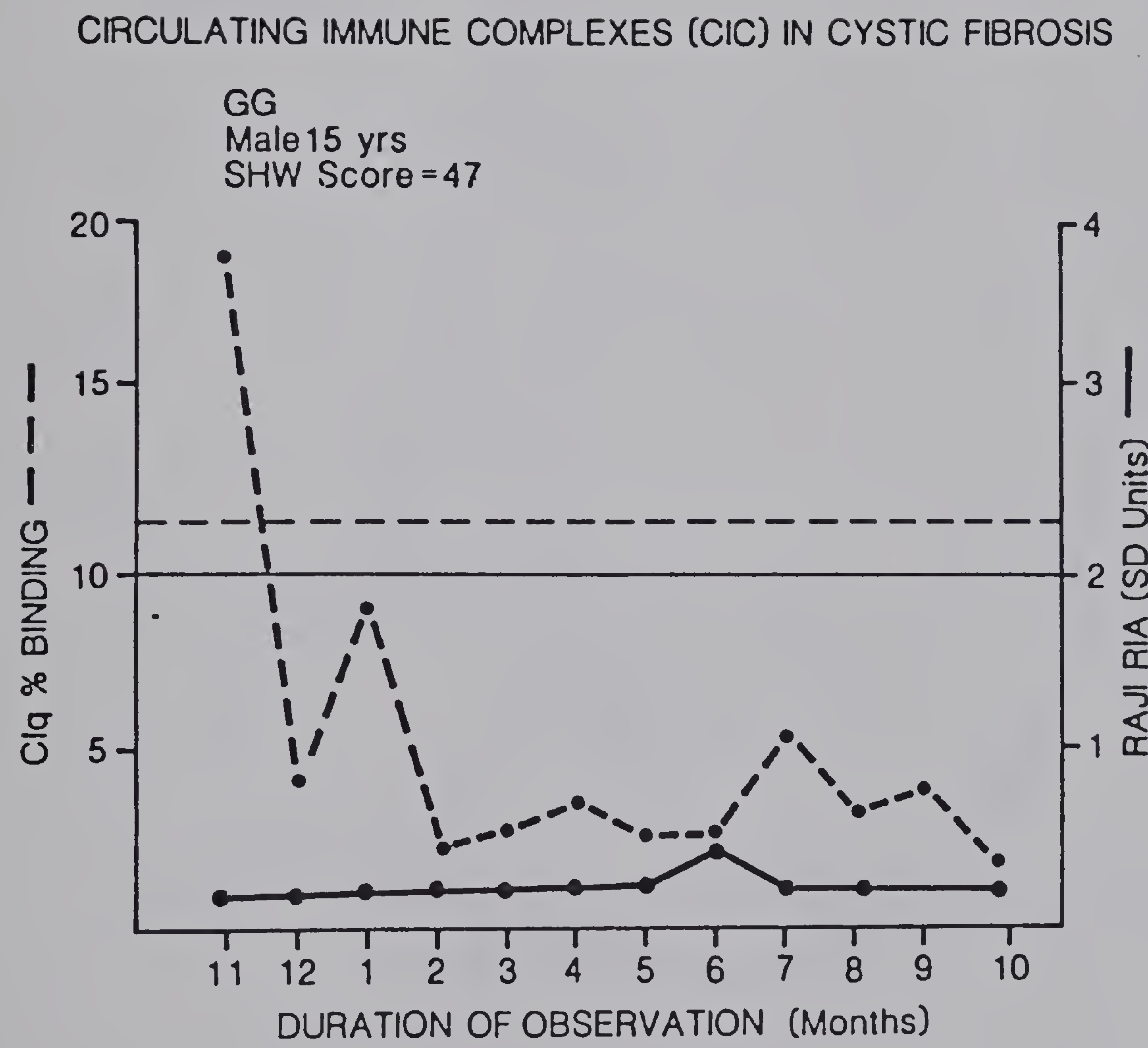

Fig. 14. CIC in Cystic Fibrosis, Longitudinal Studies by Raji-RIA and C1q-BA. Results in GG, Male 15 Years.

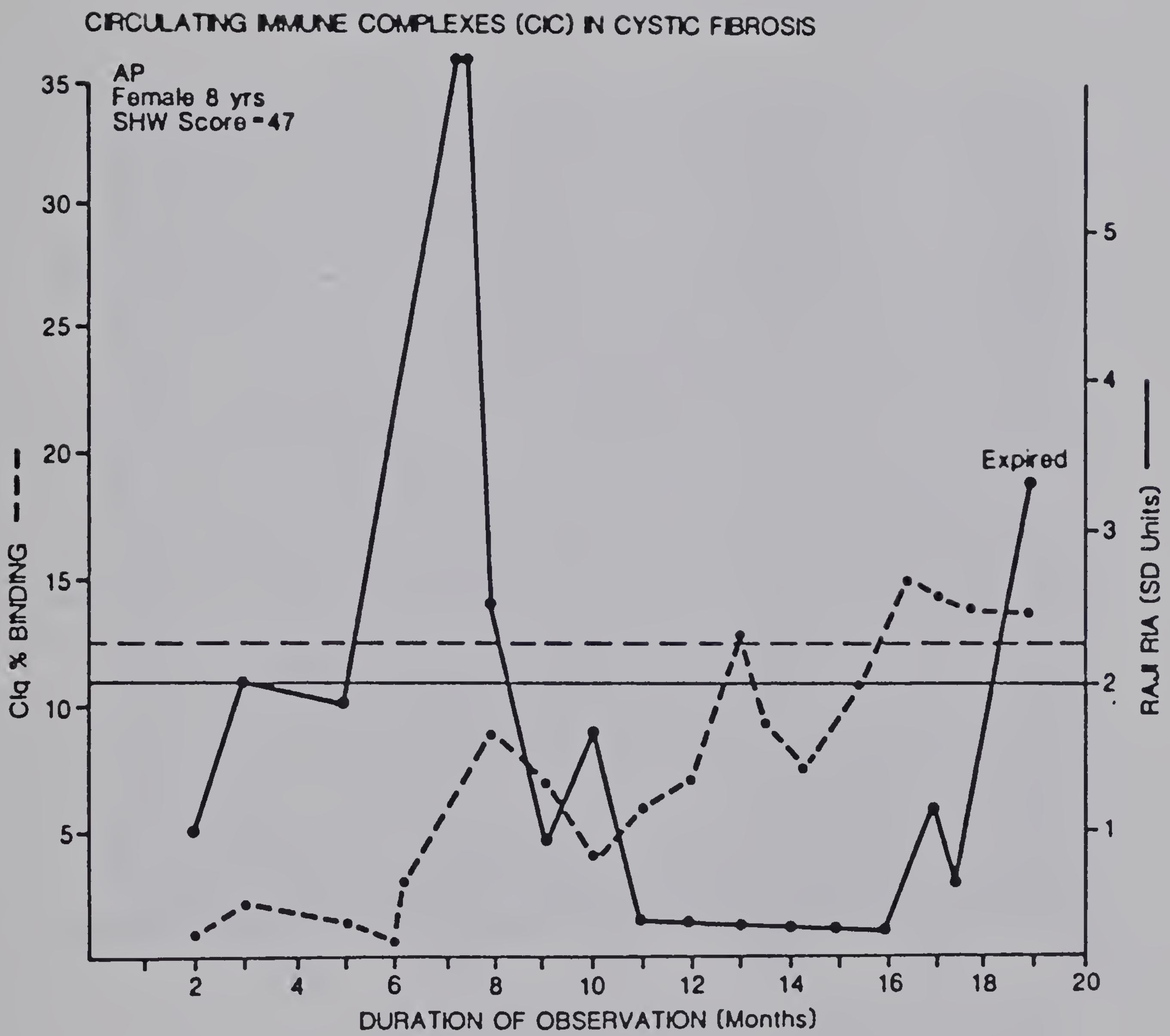

Fig. 15. CIC in Cystic Fibrosis, Longitudinal Studies by Raji-RIA and Clq-BA. Results in AP, Female 8 Years.

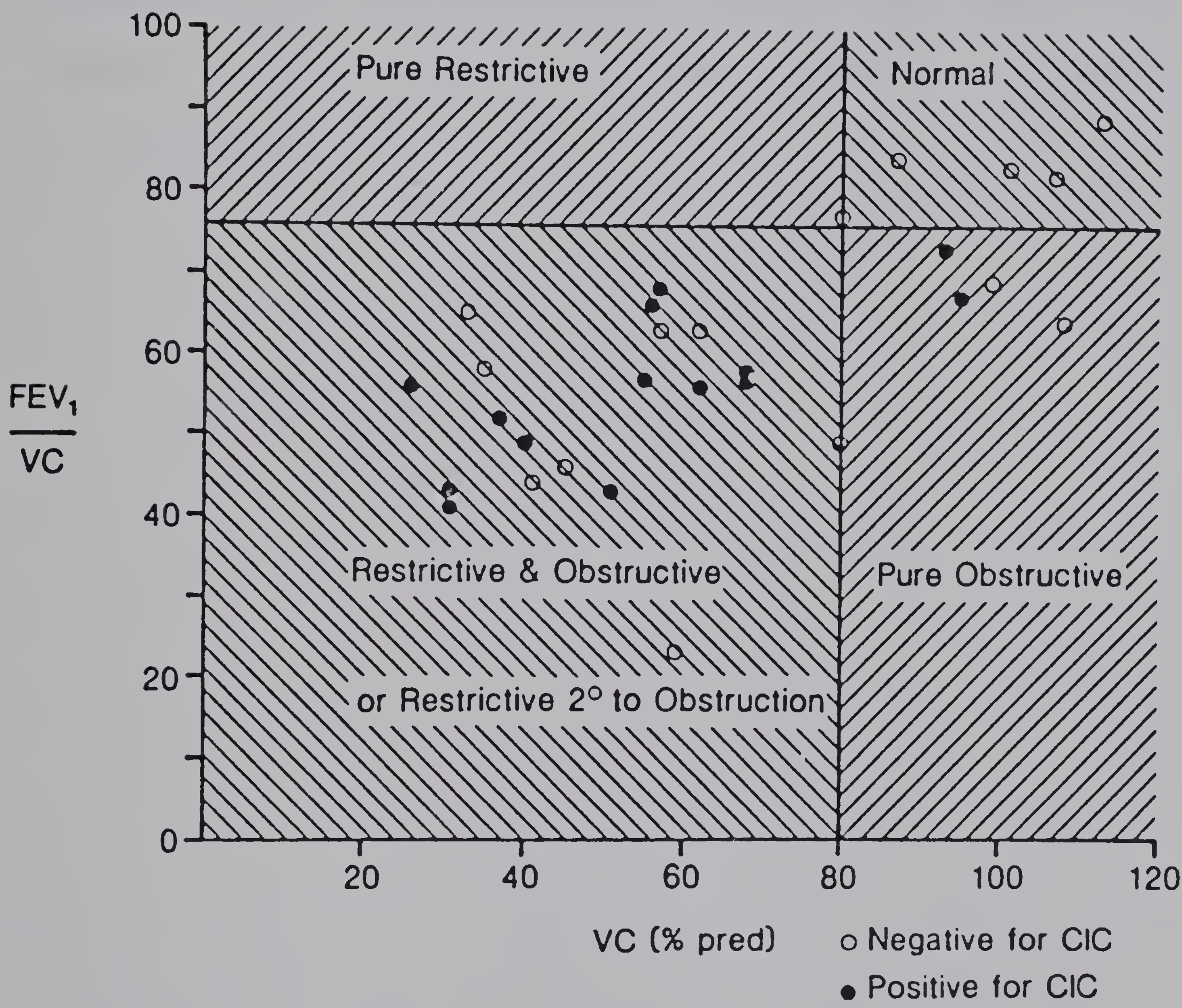

Fig. 16. Correlation of CIC Results and Pulmonary Function FEV_1/Vc in Cystic Fibrosis Patients.

restrictive (lung fibrosis) zones, as expected. No results fall in the restrictive category only. A critical look at the purely obstructive group in comparison with those with both obstructive and restrictive disease also fails to reveal that CIC prevalence distinguishes between them.

The data, therefore, is consistent with the possibility that immune complex-mediated lung damage may be occurring in at least half of advanced CF patients, but further study is certainly indicated to verify this possibility.

Chapter V: Antibodies to DNA&CIC in Patients Undergoing Long Term Hemodialysis

Damage to the formed elements of blood had been found to occur during hemodialysis, during its passage through the dialysis membrane. Nuclear debris from the damaged leukocytes has been shown on dialysis membranes using electron microscopy and other techniques (7, 112). Steinmann and Ackard (1977) described release of free deoxyribonucleic acid (DNA) into the circulation during hemodialysis (184). Circulation of free DNA and other nuclear antigens during hemodialysis might therefore lead to antibody formation. Nolph et al (137) have claimed that antibody formation to native DNA (n-DNA) and other nuclear antigens occurs in a significant proportion of hemodialysis patients in comparison to non-dialyzed rheumatoid and other control populations.

To our present knowledge no disease state other than systemic lupus erythematosus (SLE) is known to be associated with free circulating DNA and specific antibodies to native DNA. Hemodialysis would be a similar iatrogenically induced state though it is also unknown to what extent free circulating DNA and/or its antibodies could cause damage to patients on long-term dialysis, either alone or in the form of antigen-antibody complexes.

Interpretation of current methods for antibodies to n-DNA is complicated by the presence of single stranded DNA either as single stranded DNA (ss-DNA) or single stranded regions within primarily duplex molecules. The presence of ss-DNA is probably responsible for the reported incidences of antibodies to n-DNA in states other than SLE (107).

In a recent study (33) we used improved techniques to measure antibodies to ss-DNA and n-DNA amongst 48 patients in chronic maintenance hemodialysis, but failed to detect such antibodies. Our results thus differed from that of Nolph et al (137).

Besides technical differences, another important question remained unanswered, i.e. whether such antibodies were in the form of circulating immune complexes and thus accounting for the low incidence of antibodies to DNA reported in our study (33).

1. Object of the Study

a) To evaluate antibodies to n-DNA in chronic dialysis patients in comparison to non-dialyzed renal patients with advanced renal disease;

b) To measure CIC on the same serum samples by Raji RIA and Clq-BA assays; and

c) To characterize DNA - anti-DNA complexes from sera positive for CIC by DNAse digestion.

2. Patients

Treatment group

(a) Serum samples were collected from 53 patients undergoing maintenance hemodialysis. End stage renal disease was due to chronic glomerulonephritis (28 cases), polycystic kidney disease (7 cases) or chronic pyelonephritis (12 cases). Four had diabetic nephropathy. One patient developed renal failure from Alport's disease and another from Fabry's disease. Patients with known or suspected SLE or other connective tissue disorders were not included in the study. The duration of dialysis in these patients varied from two months to ten years with a mean period of 23.8 months. Fourty-nine of the patients were using a

Dow hollow fiber dialyzer and four a Gambro dialyzer. Twenty-four of the patients had previous transplants which had failed.

Control group

Serum samples were collected from 25 patients who were attending renal clinic at the University Hospital. Fourteen were suffering from active idiopathic glomerulonephritis and 11 from chronic glomerulonephritis. Six of these 11 cases had end stage disease but had not yet been dialyzed. None were suffering from SLE or other connective tissue diseases. All were under the care of nephrologists of the University of Alberta Hospital.

3. Methods

Antibodies to nuclear antigens

Antibodies to nuclear antigens were measured in all groups using standard techniques:

i) Fluorescent antinuclear antibodies (FANA): Fluorescent antinuclear antibodies were detected by exposing 1:10 dilutions of patient's serum to rat liver substrate using a standard technique. Attached antibodies were detected by a fluorescinated conjugate antigen to whole human serum (Cappel Labs.).

ii) Native DNA (n-DNA) antibodies: Antibodies to duplex DNA were measured using a synthetic polynucleotide poly deoxyadenylate-deoxythymidylate (poly dAT). This was synthesized from a reaction mixture consisting of 50 mM potassium phosphate, pH 7.5, 5 mM $MgCl_2$ and 2 mM each of dATP-deoxyadeninetriphosphate and dTTP-deoxythymidinetriphosphate in the presence of E coli DNA polymerase. This antigen was found to be 100% duplex in nature (183). Antibodies were measured by a

Millipore filter technique based on the fact that, DNA that has reacted with protein develops an affinity for nitrocellulose.

A 0.025 ml volume of undiluted test serum was gently mixed with 0.01 ml of ^{3}H-DNA solution (9.2 mg/dl). Following incubation for 15 minutes at 37°C, the mixture was passed through a 4.5 μm Millipore filter (Millipore, Canada). The test tube and filter were washed three times with 5 ml citrate buffer, pH 8.0, and once with 5 ml distilled water. After drying under an infrared lamp, the filters were placed in 10 ml DPO Toluene and counted for 10 minutes in a liquid scintillation counter.

Upper limits of normal for poly dAT were calculated as the mean ± 2 SD of binding in sera of 195 non-laboratory personnel and were found to be 10%. This method detects IgG and IgM antibodies and was found to be highly positive in SLE sera (98 out of 105).

iii) Measurement of CIC levels:

i) Raji RIA - as described in page 16

ii) Clq BA - as described in page 20

iv) Characterization of DNA containing complexes: This was done by digestion of CIC positive serum samples with DNAse as described by Cano et al (23) and Bruneau et al (21).

Briefly, 10 μg of freshly prepared (200 μg/ml) stock solution of DNAse 1 (D-4763, Sigma Chemical, St. Louis, U.S.A.) in PBS, was added to 100 μl of test serum and digestion allowed for 2 hours at 37°C. Digested and non-digested serum samples were then subjected to Raji RIA for CIC under same experimental day and conditions. Dilutions of digested and non-digested sera were adjusted similarly to a final dilution of 1:4 in PBS before adding to Raji cells. Quantitative differ-

ences in the uptake of ^{125}I anti-human IgG in the Raji-RIA between digested and non-digested serum would signify change due to DNA containing complexes.

For control evaluation three selected sera from SLE patients and three samples of _in vitro_ prepared DNA-anti-DNA complexes were studied, with and without DNAse digestion. _In vitro_ complexes were prepared in antigen excess by adding calf thymus DNA (Worthington Chemical Co, N.J., U.S.A.) to a lupus serum containing high level of anti-DNA antibody (77.4% binding).

Sera from ten healthy blood bank donors (NHS) were also run in the same experiment to give baseline uptake of ^{125}I anti-human IgG by Raji cells for the day of the experiment.

Results

Antibodies to n-DNA were detected in only two of the three dialyzed patients and one of the 25 non-dialyzed renal patients (Table 19). This low incidence of antibodies to n-DNA was confirmed by our subsequent study.

CIC levels among these sera, detected by two different methods, was positive in 17 of 53 dialyzed patients of whom 15 were positive by Raji-RIA and 3 by Clq-BA. Six samples from 25 non-dialyzed renal patients were found to be positive by both methods, 6 by Clq-BA and 5 by Raji-RIA (Table 19 and Fig. 17). Prevalence of CIC between the two groups did not differ significantly (x^2 = 0.53, $p > 0.25$).

DNAse digestion of CIC positive sera did not produce any significant change in Raji-RIA (13 of 15 positive samples) indicating absence of DNA containing complexes (Fig. 18) ($p > 0.50$).

TABLE 19

Prevalence of antibodies to n-DNA and CIC among dialyzed and non-dialyzed renal patients

Renal Patient Groups	Serum Samples (n)	Antibody to n-DNA ++ Positives x	CIC POSITIVES + Raji-RIA	Clq-BA	Combined	%
Dialyzed patients = 53	53	2	15	3	17	32*
Non-Dialyzed patients = 25	25	1	5	6	6	24*

+ = positive values representing 2 SD above mean of normal human sera (NHS) in each test

++ = detected by poly-dAT binding: > 10% binding represents positive values: 4 out of 195 non-laboratory controls and 95 out of 105 SLE sera showed positive values (33).

* p > 0.25; not significant

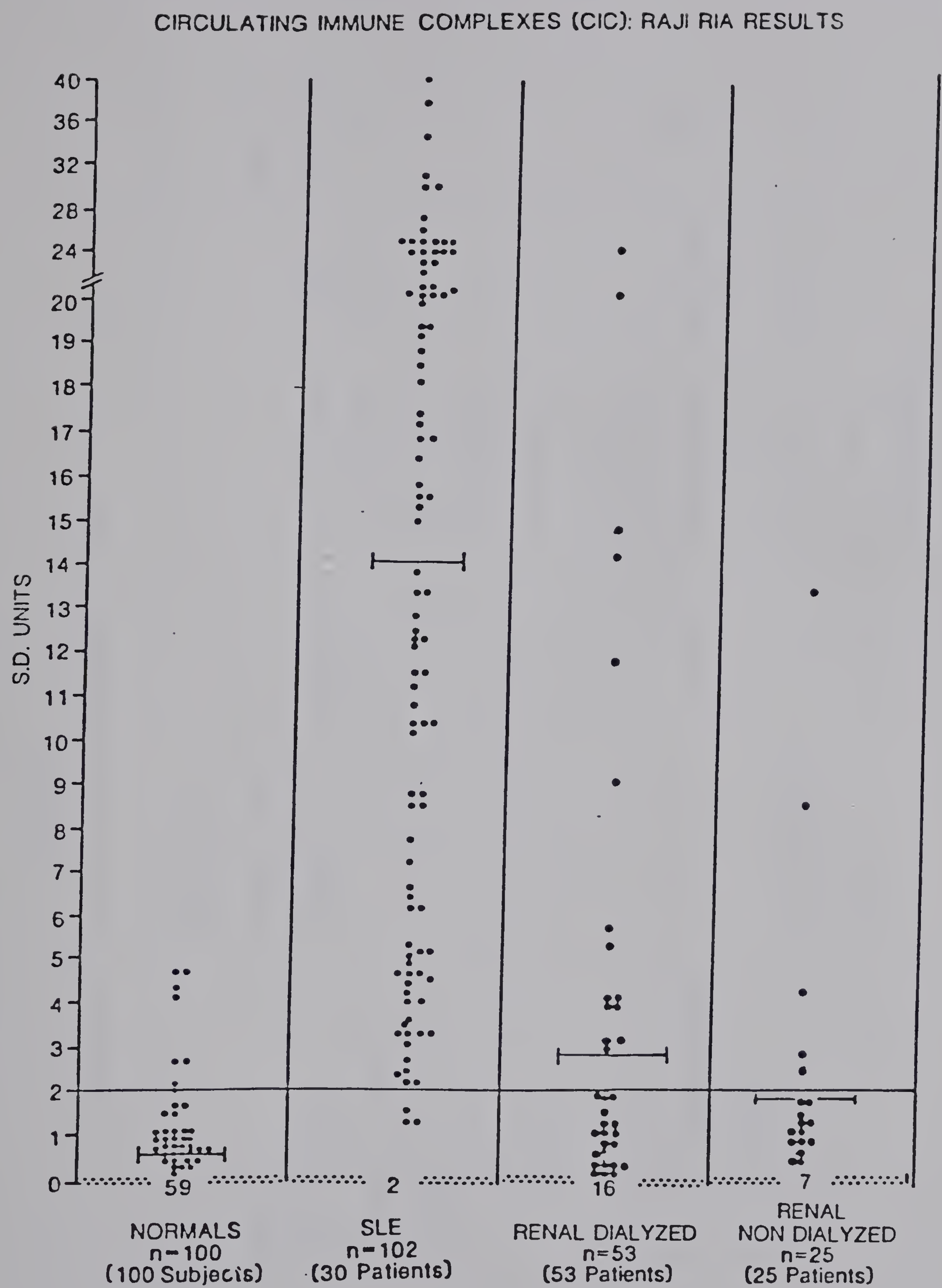

Fig. 17. Raji-RIA Results in Dialyzed and Non-dialyzed Renal Patients. Results in SLE Patients and a Group of Normal Subjects are Also Shown for Comparison.

TABLE 20

Analysis of patients with antibodies to n-DNA

Patients	Age	Sex	Cause of Renal Failure	Duration of Hemodialysis (years)	Transplants	Drug History	FANA	poly dAT + % Binding
MH*	61	F	Polycystic kidney disease	7	none	polytherapy for CRF Conjugated estrogen - 4 years antibiotics	negative	18
SE	68	M	Chronic pyelonephritis Crohn's disease	2	none	polytheapy for CRF	positive	27
OD	23	M	Idiopathic GN	Non-dialyzed control	x	multiple drug abuse Tetracycline	negative	17

+ normal values: < 10% (mean + 2 SD of normal controls)
* patients serial estimations from sera from 6 years were persistently positive for antibodies to n-DNA

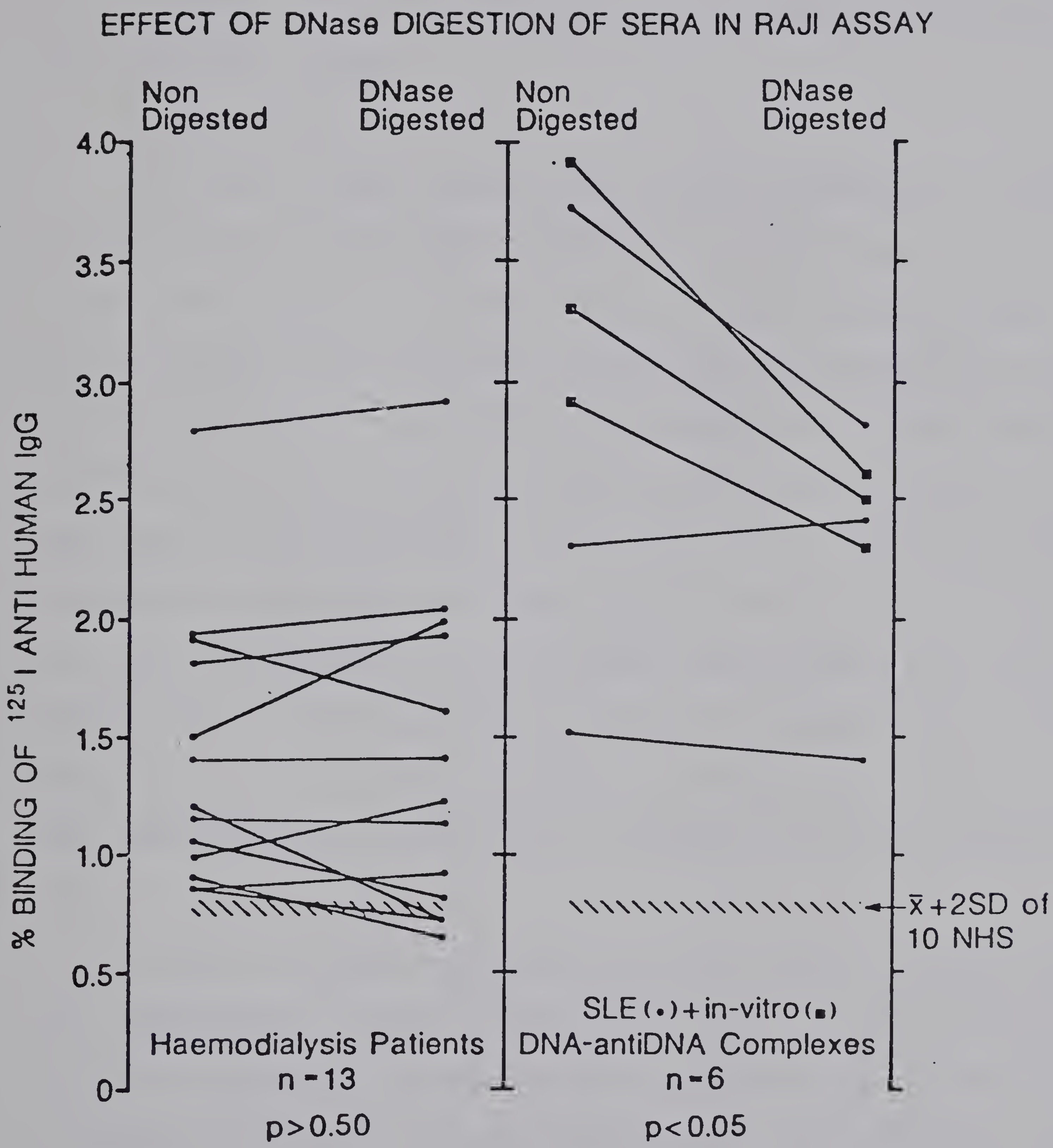

Fig. 18. Effect of DNase Digestion of Sera in Raji Assay.

Clinical details of the three patients positive for anti-DNA antibodies are given in Table 20. One (MH) was also found to be persistently positive in our previous study on serial samples (33), another (SE) had Crohn's disease.

4. Discussion

Discrepancies which currently exist on the prevalence of antibodies to native DNA can be accounted for either by the technique used to detect them or by the structural characteristics of the DNA antigen. The most widely used techniques for measurement of DNA antibodies are the Farr assay (224), the Millipore Filter Assay (56), and the immunofluorescence technique (1) using Crithidia lucilliae as substrate. While each of these techniques has individual merit, comparative studies using these three methods have shown a good degree of correlation (27, 37). The hemagglutination method of Nolph and colleagues (4) is not widely used as it may have reduced sensitivity, preferentially detecting IgM antibody, while possessing good specificity (34, 92). It has also been shown to correlate poorly with the previous techniques mentioned (31).

An alternative explanation may rest in the nature of the DNA antigen. The presence of single stranded DNA or single stranded regions within the supposedly double-stranded DNA preparation may lead to the detection of antibodies to single stranded DNA. These antibodies are not disease-specific. Their detection may account for the reported incidence of antibodies to native DNA in conditions other than SLE. Purification of DNA preparations by removal of single stranded DNA determinants has been shown to enhance specificity of assay for antibodies to native DNA (107, 166, 222). Despite attempts at purification,

some DNA preparations still contain single stranded DNA fragments (35). This problem may be overcome by the use of synthetic poly-dAT as described by Steinman (185) and as applied to the Millipore Filter Assay by Lentz et al (100). This antigen, because of its inherent biophysical properties, does not contain single stranded regions, but reacts as native DNA in radio-immune assays. Good correlation exists between antibodies to poly-dAT and native DNA.

Poly-dAT binding technique, used by us, detects both IgG and IgM antibodies. To permit both types of antibodies in the immune complexes to be detected, we selected more than one CIC assay: Raji-RIA detecting IgG bearing, C3b and C3d fixed complexes and Clq-BA detecting both IgM and IgG bearing complexes. Both assays are highly "sensitive" in clinical disease of SLE (23, 25, 97), Raji slightly higher than Clq-BA (97, 199) and also detect _in vitro_ prepared DNA-anti-DNA complexes (23, 25).

The prevalence of CIC among dialyzed and non-dialyzed renal patients was similar, 34 and 24 percent respectively, and did not differ significantly ($p > 0.25$) indicating no extra influence of dialysis technique in the genesis of CIC. DNAse digestion, also, did not give evidence that these complexes contained DNA.

It was also noted,greater prevalence of CIC by Raji-RIA in sera of dialyzed patients (15 out of 17) than by Clq-BA (3 out of 17), whereas, in non-dialyzed renal patients, Clq-BA and Raji-RIA gave similar incidence (in 5 out of 6 positive results). Thus additional positivity of Raji-RIA could be due to antilymphocytic antibodies produced by previous renal transplantation or blood transfusions. This would cause false

positive Raji-RIA assays if they attached directly to Raji cell membrane antigens.

It was noted that 24 of 53 dialyzed patients had had previous renal transplants and 25 had received previous blood transfusions. Cumulative blood transfusions among these 25 patients varied from one unit to 90 units, the average being 14.9.

Correlation between CIC positivity by Raji RIA and previous transplantation was not statistically significant ($x^2 = 1.8$, $p > 0.10$), as noted previously (Table 12), but correlation with cumulative blood transfusion was significant ($x^2 = 9.0$, $p < 0.005$), see Table 21.

As chronic dialysis patients could have persistent ADCC or CDC anti-HLA antibodies as a result of prior transplantation and/or blood transfusions, the author analyzed further the 15 Raji-RIA positive sera. This consisted of a search for ADCC and CDC antibodies against Raji cells, i.e. using ^{51}Cr-Raji cells as targets, at 37°C as described earlier. These tests are denoted as ADCC(Raji) and CDC(Raji) and comparisons were made with CDC (B) done at room temperature against a panel of 21 different panel B lymphocytes (Table 22).

It was found that 8 of 15 Raji RIA positive sera were also positive by 2-step ADCC(Raji) and 9 by CDC(Raji). Also nine of the 14 dialysis sera were positive against panel B cells in the CDC (B) test, at 20°C.

For the specific purposes of this study the of 2-step ADCC (Raji) was more suitable in detection of false positive reactions in Raji RIA because the 1-step ADCC (Raji) reaction and CDC reactions are influenced by immune complexes, if present in test sera, and because CDC reactions may also be mediated by IgM antibodies. Panel cells in the CDC(B) at 20°C have HLA antigens other than those known to be present in Raji

TABLE 21

Raji-RIA for CIC in hemodialysized patient sera:

Relation with previous transplantation and blood transfusions

		n	Raji-RIA positives	x^2	p
	Total sera	53			
	Total Raji-RIA positives	15			
I. A.	Previous transplants (Tx)	24	9	1.82	> 0.10 not significant
B.	Previous blood transfusion BTx	25	12	9.04	< 0.005 significant

TABLE 22

Patient		Duration of dialysis (yrs)	H/O blood trans-fusion	Previous Trans-plants	Raji RIA for CIC SD	ADCC(Raji) 1-step %SpCRR	Score	ADCC(Raji) 2-step %SpCRR	Score	CDC(Raji) %SpCRR	Score	CDC(Raji) + DTT Rx %SpCRR	Score	CDC(B) 21 % of panel cells+
1	VS	3	13	None	21.6	78.2	5+	61.0	5+	94.4	+	95.2		94
2	SS	2	36	1	14.4	81.2	5+	56.9	5+	97.7	+	96.8		56
3	LS	6	18	1	5.7	80.7	5+	53.2	5+	84.9	+	88.5		61
4	HV	7	18	1	8.8	41.4	4+	52.1	5+	82.9	+	87.4		72
5	BR	4	None	None	3.3	84.1	5+	42.8	4+	18.4	-	ND		0 -
6	DM	1	6	1	4.4	26.8	2+	41.4	4+	52.9	+	49.6		72
7	DG	1	57	None	14.0	15.4	+	19.1	+	92	-	ND		0 -
8	NG	3	7	1	5.3	42.9	4+	13.3	+	89.3	+	98.3		17
9	KP		24	1	24.4	44.9	4+	8.9	-	ND		ND		ND
10	GM	0.4	None	1	4.1	10.32	+	7.34	-	5.01	-	ND		0 -
11	ML^x	6	2	None	4.4	4.4	-	4.8	-	34.5	+	77.1		0 -

Continued . . .

12	SW	1	5	None	3.0	7.7	-	4.2	-	ND		ND		0	-
13	CS	1	22	1	3.9	39.3	3+	3.1	-	17.5	-	18.4		6	
14	BN		None	None	3.3	7.9	-	0.5	-	44.0	+	24.30	(+IgM)	22	
15	SE	7	26	1	11.5	47.3	4+	-2.5	-	71.6	+	52.0		72	
Total +ves					15		12/15		8/15		9/14			9/14	
Clq BA + Serums															
MH*			None	None											
PA			None	None											
SE*			None	None											

* poly DAT +ve test

x Clq BA +ve test

Table No. 22: Analysis of Raji-RIA positive 15 dialyzed serum with ADCC and CDC Raji tests and comparison with CDC(B) against panels

cells, making invalid this approach as a screening for sera likely to give false positives by Raji-RIA.

Based on these considerations it was found that 8 of the 15 Raji-RIA positive dialysis sera could be due to antibodies to Raji cells, but could also have immune complexes as well. Assuming these 8 were false positives, the true prevalence of CIC positivity among the dialyzed sera would be 9 (17%) (7 positive by Raji-RIA and 3 positive by Clq-BA) which was similar to non-dialyzed renal patients (24%).

6. Conclusions

Antibodies to n-DNA do not occur in dialyzed renal patients in comparison to non-dialyzed renal patients. These also do not exist in the form of DNA-anti-DNA circulating immune complexes.

The detection of CIC by Raji-RIA in subjects who have had much exposure to foreign alloantigens is fraught with difficulties some of which can be overcome by use of a 2-step ADCC(Raji) assay.

Chapter VI: CIC in MS Patients

1. Introduction

CIC in MS

The pathogenesis of multiple sclerosis (MS) remains undetermined though the association of several immunologic abnormalities suggests an autoimmune aspect in this disease. These include abnormal cell-mediated responses to nervous tissue antigens (159, 217), abnormal suppressor cell populations (10, 57, 168), increased incidence of anti-DNA (174), anti-measles (4), cold reacting anti-lymphocytotoxic antibodies (96, 172), and altered responses to viral antigens (119, 160). Recently circulating immune complexes (CIC) have been described in MS sera with variable frequency and without any correlation to disease activity (38, 59, 80, 81, 171, 186, 203). These studies are difficult to interpret because they employed different CIC assays in small numbers of patient samples. Moreover, it was established by the WHO multi-centre study that the frequency of CIC detection in a specific disease entity depends upon the type of assay used (97). Nevertheless, the demonstration of IgG and complement in the plaques of MS lesion in the brain (227), activation of serum and CSF complement in active MS (115), increased production of IgG in CSF (151) and detection of CIC in MS sera all suggest that immune complexes might play a role in the pathogenesis of MS lesions.

Experimental allergic encephalitis (EAE) has many pathological and immunological features which resemble human demyelinating disease (MS). This autoimmune disorder of the central nervous system is produced in animals (monkeys, pigs, rabbits, guinea pigs, rats, and mice) by inoculation with myelin basic protein (MBP) in complete Freund's adjuvant

(151). Cell-mediated and humoral immune responses to the sensitizing antigen occur concomitantly with development of experimental disease (62, 121). In MS, investigators have reported cell-mediated immunity to MBP *in vitro* (88, 105) and antibodies to myelin or crude CNS antigen preparations (106, 165).

Primary myelin destruction occurs in MS; and MBP or a fragment of MBP is reported in CSF of patients with the disease (29, 215) and levels may fluctuate with disease activity (28, 93). There is also evidence for individually specific formation of IgG oligoclonal bands in 90% of CSF of MS patients, by agarose gel electrophoresis, which may be sufficiently specific to "fingerprint" a particular MS patient, only the levels varying in different phases of the disease (170, 83). This excess of immunoglobulin is produced in CNS (138) and sometimes contains antibodies to one or more viruses (200, 207) although most of the oligoclonal IgG bands cannot be removed by absorption with viral antigens (207), representing antibodies to other unknown antigens. Older techniques failed to show antibodies specific to MBP (138, 151), but recently IgG antibodies specific to MBP have been demonstrated by solid phase RIA in the CSF of MS patients (149). It would seem therefore that, as a part of defective immunoregulation in MS (mediated possibly by viruses), there may be repeated damage to myelin sheaths with release of MBP or fragments of MBP and autoantibodies could be produced against them, and immune complexes could form between MBP and anti MBP.

Demonstration of such complexes would strengthen the hypothesis of autoimmunity in MS but would not explain their role in immunopathogenesis. Reports of immune complexes detected in MS sera and CSF give

no information of the antigens in such complexes (38, 59, 80, 81, 171, 186, 203).

Objectives of Studying CIC in MS Patients

a) Because of wide variation in reports of the prevalence of CIC in MS patients as well as in their relationship to disease activity, the author wanted to re-examine these questions using three CIC methods each based on differing biologic principles. This would be done on a large number of samples and evaluated in a single blinded fashion.

b) Secondly, an attempt was made to characterize the antigenic moiety in CIC eluted from serum of MS patients as to whether or not it contained MBP. The possible significance of this antigen has already been discussed.

2. Materials and Methods

Patients and Controls

a) MS patients: Dr. K.G. Warren was responsible for the diagnosis of MS and subsequent allocation into four clinical subgroups. At the time of serum sampling, he did this in ignorance of the CIC results. The clinical subgroups were:

1) acute relapse, with or without optic neuritis;
2) progressive - where the disease is progressing year by year without any clinical evidence of recovery, some progressing to death;
3) remission - samples taken within a month of clinical recovery from an acute relapse; and
4) stable - as progression year by year but with stable neurologic deficits.

252 serum samples from 254 patients were analyzed. Amongst these were 111 females. Patients' ages ranged from 16 to 73 years (mean = 39 years).

b) Neurologic Controls: Sera from 34 patients suffering from neurologic disorders other than MS were used as one control group. Patients in this group included several types of acute and chronic neurological disorders, namely Guillian-Barre Syndrome, Bell's Palsy, acute disseminated encephalomyelitis, myasthenia gravis, acute meningo-myelitis, brain tumours, acute cerebrovascular accidents.

Sera from MS and neurologic controls were prospectively collected, divided into aliquots, coded and stored at -70° by KGW. Coded samples were assayed for CIC within 8 weeks of collection.

Distribution of patients in the MS subgroups and neurologic controls are shown in Table 23.

c) Normal Controls: Serum samples from 116 healthy blood donors were simultaneously examined as normal controls.

CIC Assays: Raji-RIA, Clq-BA, and Bovine Conglutinin binding assay was used.

Bovine-conglutinin binding assay: Technical details of the assay have been described by K.V. Johny et al (85) and is currently in use in our laboratory. Normal values were 3.0 ± 2.9 as mean and 1 SD. Results above 8.8 were considered abnormal and expressed as % binding of ^{125}I bovine conglutinin.

Isolation and Characterization of CIC From Raji Cells

This was done by adsorption of CIC from serum of MS patients onto Raji cells followed by acid elution with isotonic citrate buffer using

techniques described by Theofilopoulous et al (196). Details are given in Appendix 3 and 5.

a) Raji Elution: Briefly 30 x 10^6 Raji cells were incubated at 37°C for 45 minutes with 200 μl of test serum and were then washed three times by wash media. Raji cells with adsorbed CIC on their surfaces were then incubated for 7 minutes in isotonic citrate buffer (containing 1% BSA) at pH 2.8 to 3.0. In previous kinetic studies we found that incubation beyond 7 minutes would decrease Raji cell viability (data not included). Cells were then centrifuged at 500 g for 7 minutes at 4°C and supernates were collected, coded and passed on for SDS-PAGE analysis.

b) SDS PAGE and RIA for MBP: Coded samples from acid elutions were passed directly onto % SDS polyacrylamide gels. Subsequent gel electrophoresis separated the peaks of MBP which were then identified, after elution from gel, by Dr. S. Sutherland using techniques well established in Dr. McPherson's laboratory and described in detail in Appendix 5.

MBP or its fragments are detected, according to their molecular weights, in 3 different peaks: (a) Peak I = "MBP" - molecular wt 18,400, (b) Peak II = "fragments of MBP" - molecular wt < 18,400 and, (c) Peak III = "high molecular wt MBP" - molecular wt > 18,400 = "Big BP"

Quantitation of the MBP is expressed in ng/ml.

Statistical Analysis

(i) The author used the large sample approximation of Irwin Fisher Exact Test. Although the numbers in MS subgroups were not equal the minimum number of patients in each group was 20. Therefore a large

sample approximation to the hypergeometric distribution to calculate *Z* scores between groups of CIC results, by Irwin Fisher Exact Test was used. Since it is expected that scores of affected populations should exceed those of unaffected populations, one tailed tests were used. Therefore any calculated *Z* score which equals or exceeds 1.65 represents a difference between groups which is significant at an alpha level of 0.05. In all cases, the *Z* score was calculated by subtracting the values of the one group from the other, positive scores signifying a higher value for the second group.

(ii) Chi-Square test was used for testing for association between HLA antigens and CIC positivity.

3. Results

Prevalence of CIC in MS

Table 24 compares three different CIC methods. CIC detection was more prevalent by Raji assay than Clq or Bovine conglutinin assays. Overall incidence of CIC amongst these 272 samples by combination of three methods is found to be 34.9%.

As CIC were detected most frequently by Raji assay, the author compared the incidence of Raji positive CIC in the different clinical subgroups of MS patients with other neurologic disorders (neurologic controls) and healthy blood donors (normal controls) as shown in Figure 19. 8.6% of neurologic control sera were positive by Raji RIA in comparison to 29.4% amongst MS patients. Three positive sera in neurologic controls were from patients with myasthenia gravis, without thymoma (2 cases) and a patient suffering from mumps meningitis. Statistical evaluation of the comparative incidences of CIC between different subgroups of MS patients and neurologic control group is shown in Table 25.

TABLE 23

Details of the Clinical State of the Patient and Control Groups in MS:

Patients and Controls	No of Patients	No of Samples
A. MULTIPLE SCLEROSIS:		
a) Acute relapse	74	81
b) Progressive	88	96
c) Remission	66	69
d) Stable with deficits	26	26
TOTAL	254	272
B. NEUROLOGIC CONTROLS:		
Viral encephalitis	3	3
Herpes simplex virus encephalitis	3	3
Herpes Zoster (thoracic)	2	2
Herpes Zoster ophthalmicus	2	2
Vestibular neurositis	2	2
Mumps meningitis	1	1
Measles meningoencephalitis	1	1
Bell's palsy - acute	2	2
Guillain Barre syndrome	4	4
Myasthenia Gravis	2	4
Cerebral atrophy with tremor	1	1
Parkinson's disease	2	2
Cerebrovascular accidents (acute)	1	1
Meningioma	1	1
Post polio muscular atrophy	1	1
Atypical facial pain	1	1
Headache NYD	2	2
Depression	1	1
C. HEALTHY BLOOD DONORS	116	116

TABLE 24
Incidence of CIC in Multiple Sclerosis (MS) Patients
by Three Different CIC Assays

MS Disease		Raji		Clq-BA		Conglutinin-BA		CIC	
Subgroups:		+	%	+	%	+	%	+	%
Acute relapse	n = 81	27	33.33	14	17.28	4	4.9	32	39.50
Progressive	n = 96	29	30.20	14	14.58	4	4.16	37	38.54
Remission	n = 69	18	26.08	7	10.14	4	5.79	18	26.08
Stable with deficit	n = 26	6	23.07	5	19.23	0	X	8	30.76
GROUP TOTAL	272	80	29.41	40	14.70	12	4.4	95	34.92

\+ = positive results in CIC $\geq \bar{X}$ + 2 S.D. in each assay

CIC* = cumulative positivity when one or more assay is positive in individual serum sample

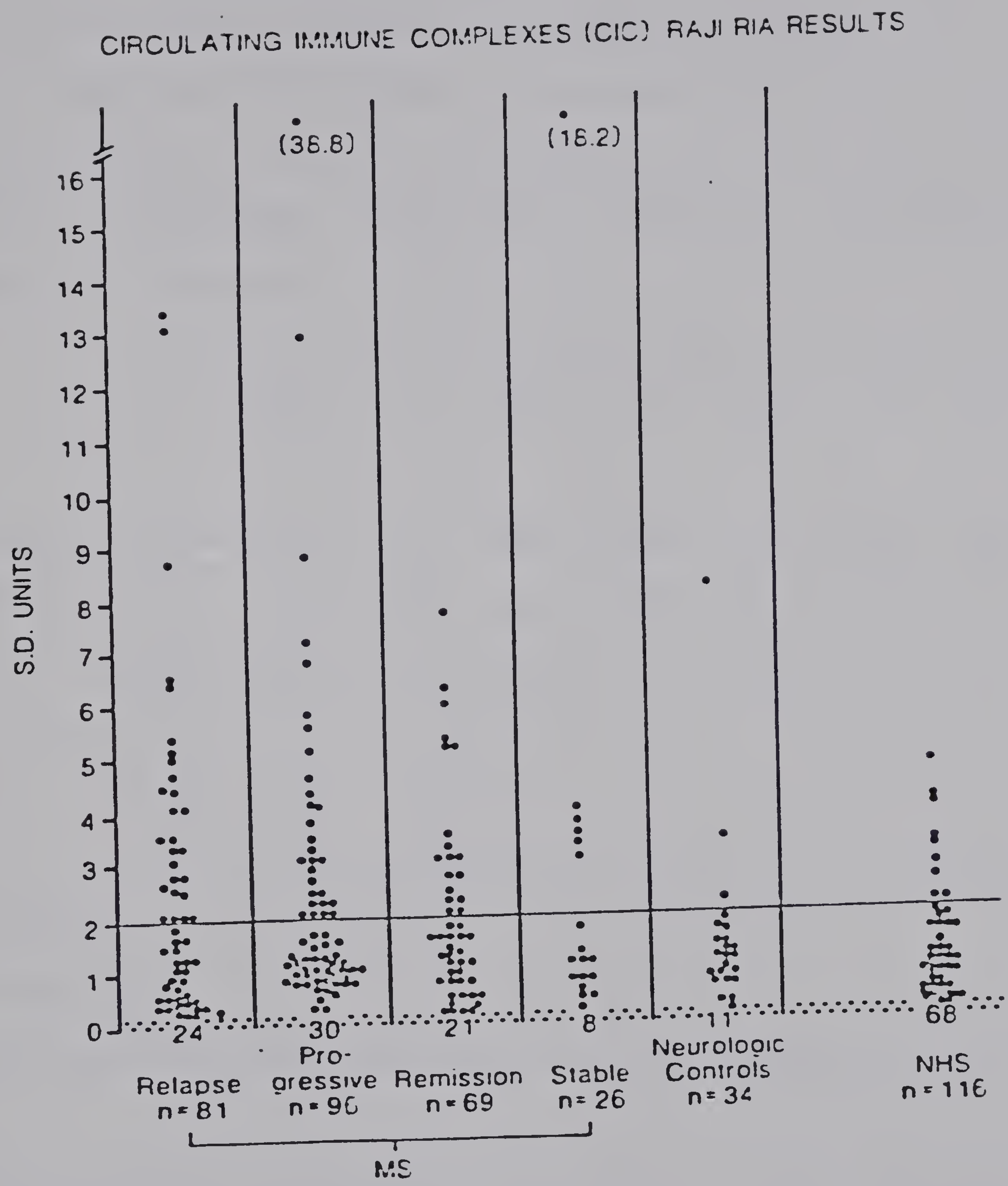

Fig. 19. Figure showing the prevalence of circulating immune complexes (CIC) amongst multiple sclerosis (MS) patients and controls as detected by the Raji cell assay (Raji-RIA). Results are expressed in S.D. units above the mean (see Methods). The values around the normal mean are indicated in the shaded areas at the base of the graph.

TABLE 25

Comparison of Incidence of CIC Positivity Amongst Subgroups of MS Patients and Neurologic Controls"

MS Disease Subgroups	Neurologic Controls		Stable with deficit		Remission		Progressive	
	Z	P(Z)	Z	P(Z)	Z	P(Z)	Z	P(Z)
Acute Relapse	2.48*	.007*	0.1+	.50+	0.965+	.17+	0.44+	.33+
Progressive	2.31*	.011*	0.30+	.48+	0.579+	.28+	X	X
Remission	1.87*	.031*	0 +	.99+	X	X		
Stable with deficits	1.27+	.102+	X	X				

* = significant

\+ = not significant

Z = scores from large sample approximation of Irwin Fisher Exact Test

P values are obtained from standard tables from Z to P and given as P(Z)

" Detailed CIC data is given in Figure 19

Patients in acute relapse and progressive disease differ very significantly from the neurologic controls. Patients in remission also differed significantly from neurologic controls, but to a lesser extent than the patients in acute relapse and progressive disease. This may indicate that, although clinical remission has occurred, biologic remission may not yet have occurred. Patients who are stable year after year, with neurologic deficits, did not differ significantly from the neurologic controls. Thus our results show a positive relationship between CIC and disease activity in MS, although there is no significant difference between any single MS clinical subgroup and any other subgroup.

Characterization of CIC by Raji Cell Adsorption and Acid Elution

A total of 26 MS sera were used for adsorption and elution from Raji cells and subsequent characterization of MBP or its fragments. Seventy-five percent of the MS samples were positive for MBP but correlation with CIC positivity by Raji-RIA in original serum was not significant ($p > 0.10$) (see Fig. 20, 21, and Table 26).

Serum samples from non MS neurologic controls which were subjected to the same analysis included five from non-MS demyelinating diseases and one sample of myasthenia gravis without thymoma (Table 27). Out of all of these controls, only one was positive for MBP, a case of herpes virus encephalitis.

Other controls consisted of 6 highly positive sera for CIC by Raji RIA from 4 SLE and 2 SBE patients - disease conditions where chance of demyelination would be remote. None of these samples were positive for MBP (Table 27).

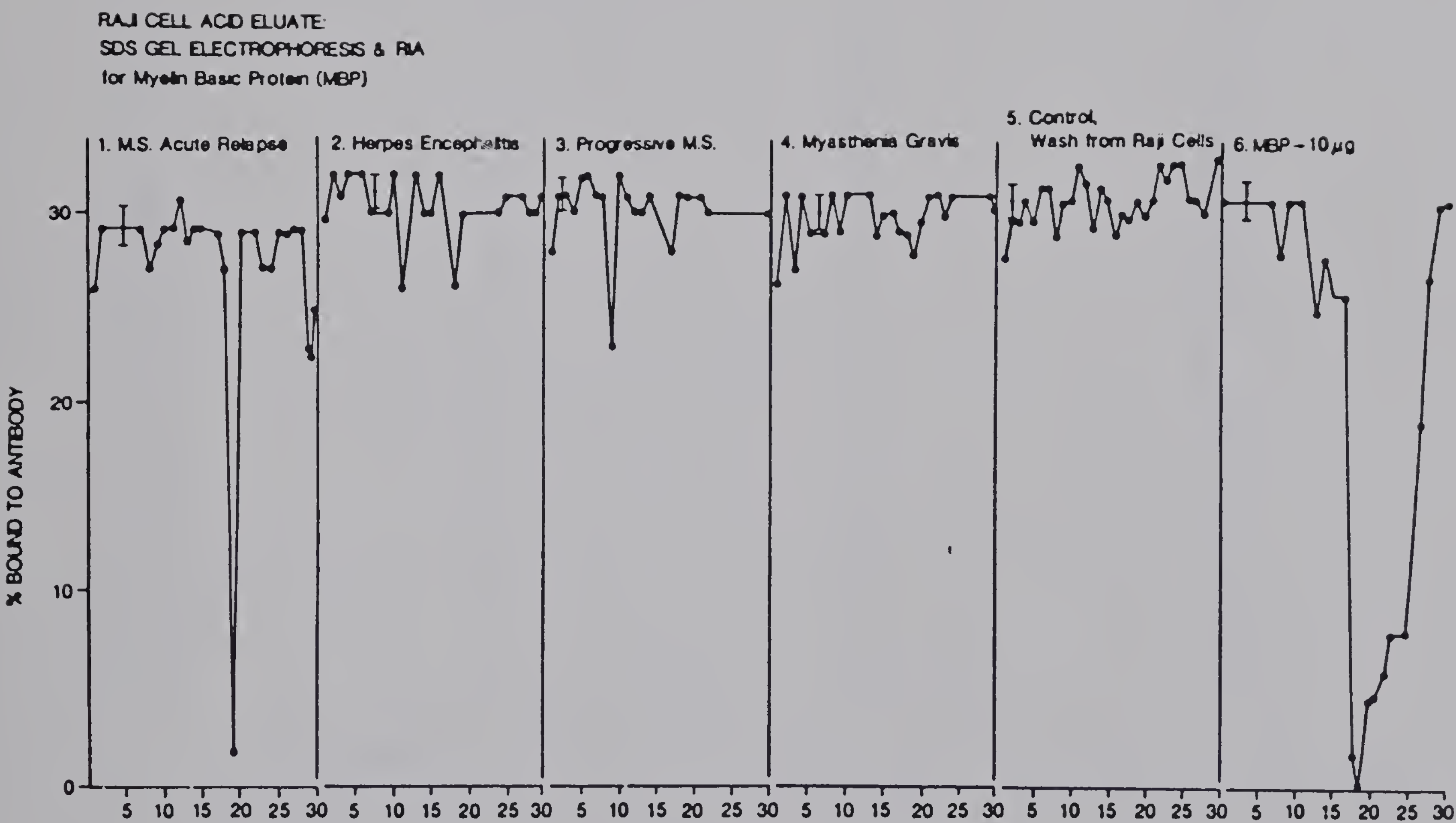

Fig. 20. Representative Myelin Basic Protein (MBP) Peaks as Detected by RIA for MBP Following Elution of SDS-PAGE of Raji Cell Acid Eluates.

TABLE 26
Quantitative Results of Myelin Basic Protein (MBP) in M.S. Sera, Isolated From Raji Cell and Corresponding Results by Raji-RIA for CIC

M.S. Sample Code No.	MBP* Equivalents (ng/ml)	BPI** (ng/ml)	Raji-RIA for CIC (S.D. units)
42	24	14	4.7
52	33	0	8.6
281	6	2	1.8
331	1	1	4.4
CSF	0	0	0
CSF	0	0	0
335	4	1	6.4
113	0	0	4.4
127	0	0	0
424	0	0	8.5
571	1	1	5.1
564	1	0	0.1
325	1	0	0.8
309	0	0	6.0
330	3	0	3.8
292	4	1	2.4
110	0	0	0
533	0	0	3.7
293	3	1	0.3
337+	2	1	3.1
355+	0	0	0
425+	0	0	2.8
437+	6	5	1.4
363+	1	1	0.4
388+	3	1	1.5
491+	0	0	1.8

+ = Same patient
* = Cumulative results of the three MBP peaks quantitated by RIA (see text)
** = Quantitative results of MBP peak 1 only.

TABLE 27

Results of Raji Cell Acid Elution of MBP: Control Groups

	MBP equivalents ng/ml	Raji-RIA for CIC S.D.
a) Neurologic controls:		
1. Herpes virus encephalitis	7	0
2. Herpes virus encephalitis	0	1.6 -
3. Guillain Barre syndrome	0	0.6 -
4. Guillain Barre syndrome	0	1.1 -
5. Viral encephalitis	0	0.1 -
6. Myasthenia gravis	0	8.1 (+)
b) Non neurologic controls:		
1. SLE (active disease)	0	7.7 +
2. SLE (active disease)	0	27.6 +
3. SLE (active disease)	0	5.2 +
4. SLE (active disease)	0	8.8 +
5. Subacute bacterial endocarditis (SBE)	0	24.8 +
6. Subacute bacterial endocarditis (SBE)	0	7.4 +
c) Other controls:		
1. MBP in acid buffer	200	
2. MBP ∞ MBP *in vitro* complex NHS	6	
3. NHS	0	
4. CSF (MBP RIA 18 ng/ml)	0	0 -
5. CSF (28 ng/ml)	0	0 -
6. Raji washings	0	x

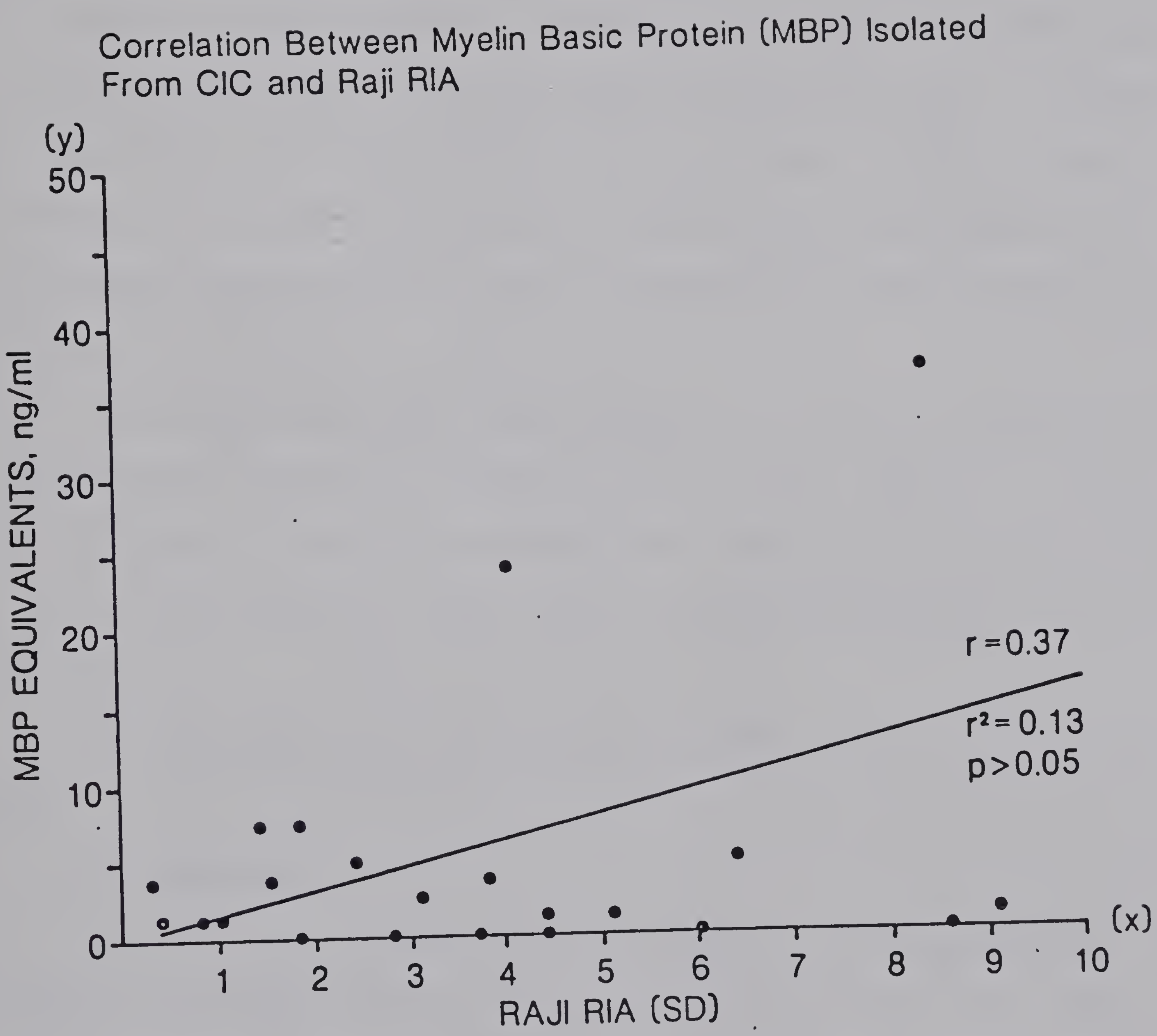

Fig. 21. Correlation Between Myelin Basic Protein (MBP) Isolated From CIC and Raji-RIA

Positive controls were prepared *in vitro* by making complexes of BP-anti-BP which were added to NHS, which was positive for MBP after acid elution from Raji cells but NHS alone was not (Table 27). MBP added to the buffer alone was recovered from the gel without any denaturation. This indicated that the acid buffer (isotonic citrate buffer at pH 2.8 - 3.0) did not interfere with isolation of MBP in this system.

Raji cells were incubated in wash media and washed three times and treated similarly with acid buffer wihtout adding human sera. Supernate of these Raji acid eluates did not show the presence of MBP on repeated testing indicating that experimental conditions would not cause MBP to be released from Raji cells. This is referred to in Table 27 and Fig. 20 as Raji washings.

Samples containing high concentration of BP (but not complexed with antibody) were tested for binding to Raji cells and subsequent elution. Two CSF samples, from MS patients which had high in MBP by RIA did not show any MBP (Table 27) in Raji eluate. Raji-RIA was negative in both the samples.

Thus the results show that CIC in MS contain MBP, and controls do not.

4. Discussion

Table 28 lists the incidence of CIC in MS in recent publications compared to our own results. Most studies show serum CIC to be present in a significant proportion of MS patients, though the incidence varies. Our data, the largest series of patients evaluated to date, also confirm this. Not only does the incidence of CIC vary, but so do the methods used for their detection (see Table 28). All authors but one do not find correlation between serum CIC and disease activity, though one

TABLE 28

CIC Results in Multiple Sclerosis Patients:

Comparison of Literature with Present Study

Authors	CIC Methods	MS		NHS		OND	
		n	% +ve	n	% +ve	n	% +ve
1 Tachovsky et al(1976)[186]	Raji RIA	67	49	27	15	55	21.8
2 Jacques et al(1977)[180]	Clq-PEG	38	29	35	0	35	8.6
3 Goust et al(1978)[159]	Clq-PEG	19	14*			16	15*
4 Jans et al(1980)[181]	CCT, TAT	53	40	-	-	ND	-
5 Deicher et al(1980)[138]	Clq-PEG	98	26.5	118	2.5	42	14.3
6 Schockett et al(1980)[171]	Clq-PEG,	48	8.3	200		ND	
	Raji-RIA	siblings		7	0		
7 Troullas et al(1980)[203]	Clq-PEG						
	(sporadic)	77	43	67	12	ND	
	first degree relatives			48	52		
	(familial)	9	0				
	first degree relatives			26	0		
8 Present study (1981)	Conglutinin-BA, Clq-PEG, Raji RIA	272	34.9	116	7.7	34	8.8

OND = Other neurologic disorders
NHS = Normal human sera
ND = No data
* cases, not %

notes some relation with "acute bouts of the disease" (38). Comparison of these studies is made difficult by: diversity of assays employed; the small number of patients studied; and lack of standardization of criteria for disease activity.

Assays for CIC are known to differ in sensitivity and specificity, probably reflecting the biologic diversity of the complexes, as each depends on different CIC characteristics (97). It is therefore important to use a battery of tests to cover different characteristics when studying unknown CIC. In our initial studies, therefore, we used three different methods: a) Clq-BA: detecting complexes with IgG and IgM class and activating complement via the classical pathway; b) Bovine conglutinin-BA: detecting complement-activating complexes with bound C3bi; and c) Raji cell RIA detecting IgG type complement-activating complexes, predominantly by bound C3b (194). In MS patients the incidence was 4.4% with conglutinin binding, 14% with Clq-BA and 29.4% with Raji-RIA. Previously, Tachovsky et al (186) noted a high incidence of complexes in sera of MS patients using Raji assay, but they made no comparative observations with other CIC methods.

A proportion of MS patients develop anti-lymphocytotoxic antibodies (96, 172) which, if capable of combining with antigenic determinants on Raji cells, might be a cause of false positive results with this assay. Although we have not specifically tested our MS sera, we believe false positives would be minimal as the anti-lymphocytotoxic antibodies described were cold reactive (96, 172).

Our neurologic control group had a similar CIC incidence to normal controls and differs significantly from sera of patients in acute MS relapse or with progressive MS disease, but not from the 26 samples from

patients with stable MS. This demonstrates a tendency for association between CIC and disease activity in MS. Patients in clinical remission also had higher incidence of CIC and differed significantly from the neurologic control group. This may reflect the fact that, although patients may be clinically improved within a month or so of an acute relapse, some aspects of biological remission have not yet occurred. This conjecture would justify the use of serial CIC measurement as a guide for evaluating completeness of remissions after acute relapse.

Some pathological findings in active MS lesions favour cell-mediated and immune cmplex (IC) mediated injury (10, 115, 159, 217, 227). Immunoglobulins are found in the walls of blood vessels close to areas of MS inflammation, areas which will subsequently become areas of demyelination (189, 227). However the precise mechanism of IC mediated injury is not known. Our data, showing a degree of correlation between serum CIC and MS activity, was a stimulus for isolation of complexes and examination for MBP content.

These findings support a concept of autoimmunity in MS, similar to that in experimental allergic encephalitis in animals, but does not define the role of CIC in pathogenesis.

Conclusions

1) There is an increased prevalence of CIC in MS patients in the active phase of the disease;
2) Raji-RIA reveals a higher incidence than C1q-BA and Conglutinin-BA;
3) Some complexes eluted from Raji cells contained MBP as an antigenic component, in contradistinction to CIC eluted from sera of other diseases.

Chapter VII: General Discussion

1. Developmental Aspects

Antigen non-specific methods of immune complex (IC) detection have general limitations: i) wide variation occurs in the same disease when comparison is made between different methods or by the same methods in different laboratories; ii) they lack specificity; iii) it is usually not possible to isolate antigens from CIC for animal immunization to produce antisera or to establish pathogenicity by other means.

Some of the drawbacks of CIC assays lie not so much in techniques but in the kinetics, in vivo, of IC formation and clearance associated with changes from antigen (ag) excess to antibody (ab) excess, or from smaller to larger complexes, or from one antibody class to another. Different CIC methods vary in their ability to detect IC of different ab class, size and zone of IC formation (Table 1 in Chapter I). It would not, therefore, be expected that any single CIC test could fulfill the task of detecting all forms of CIC. Other reasons for wide variations in results between methods are due to: i) lack of commercially available potent and stable biological reagents such as human C1q, Rheumatoid Factor (RF), Bovine conglutinin, etc.; ii) lack of uniformity in the panel of tests in individual disease states and; iii) lack of assay standardization and quality control. A WHO study in 1978 (97) compared 18 different CIC methods in 300 pathological sera. Only six of 18 methods were "sensitive" enough to be used further. Raji-RIA and C1q-BA were two of the six best methods recommended.

The WHO study (97) also recognized the problem inherent in the use of heat-aggregated human gammaglobulin (AHG) as the standard. Standard AHG preparations were found to be quite unstable (180), as also noted by

others (90, 116, 147, 205). We also found this to be so. Dissociation or self aggregation of AHG molecules occurs on storage even at room temperature for 15 minutes (90). This leads to unreliable standard curves. Aggregates can be stabilized by adding BSA (90) or by alkali treatment (210), but ^{125}I labelling was used to reveal stability of IgG aggregates (90). Recently another method for checking aggregate size has been described (116) which claims that BSA addition is not necessary if batches of IgG are carefully selected but no data were given on storage beyond several months. We, on the other hand, have shown that serum from a SLE patient (LJ) run in serial dilution gave better standardization than AHG. We also demonstrated that aliquoted LJ sera could be used for a period of one year without change in reproducibility (Table 6, Chapter III) but we have not compared LJ sera titration with any of the stable AHG preparation made with BSA.

Even after selection of a supposedly stable batch of AHG, other critical problems remain. Heating of human IgG causes aggregation of 20% of the total content into macromolecules varying from 40-400 S in Svedberg Units (147, 208). Concentration of AHG may be similar in different batches but these would give no assurance of uniformity in aggregate size in different batches (90). Aggregates of standard size and lattice structure are critical for IC determination (eg. C activation, RF binding, etc.) and determine sensitivity in different assays. Therefore, the use of AHG for standardization is scientifically unacceptable when one realizes that such standards contain different polymers of aggregate binding as well as concentration. Mere expression of concentration without size specification is misleading. For this reason our use of in vivo LJ sera and reference NHS is better, but the ideal

solution would be to have in vitro prepared stable IC or AHG aggregates of defined polymeric size. This is currently under investigation in a second WHO study (216).

After we had modifed (improved) standards for our Raji assay, we noted that this assay was more often positive than other PEG dependent assays done in our laboratory, as is also reported in other comparative studies (25, 45, 84, 97) and referred to, in the first WHO study, as higher "sensitivity" of the Raji assay (97). We believe that this is due to the presence of various types of receptors on Raji cells which are also responsible for their "broad reactivity", as they would pick up all types of complexes.

The chief limitation of the Raji-RIA, like any other CIC method based on a cell line, is: 1) the time and expense required for maintenance of the cultured cells and, 2) that such cells will react with antibodies directed against their membrane antigens to give false positives. Although maintenance of cultures is expensive, it ensures the purity of the biologic material. This is not the case with other methods of CIC assay which lack uniform preparation of reagents (eg. human C1q or RF). Regarding the second limitation, we have data to show that antilymphocytic antibodies are not an important cause of false positive Raji assay when the conditions are known and defined. Using the same incubation temperature (37°C) as for Raji-RIA, we used the specific target (^{51}chromium-labelled Raji cells) by ADCC(Raji) and CDC(Raji). We found no significant increase in warm reacting anti-Raji membrane antibodies of IgG class in SLE sera. For accurate assessment of CIC in transplant rejection sera or multiparious or multitransfused sera, ADCC(Raji) and CDC(Raji) are important to exclude false positive

reactions (Chapter III, Section 5), however it was believed that false positivity to Raji assay by antilymphocytic antibodies has been overemphasized in literature by noting reactions against panel B cells at different thermal incubation but not using Raji cells as targets at 37°C. Recently Maini et al (1980) (76) have indicated Raji assay may detect antinuclear antibodies of different types, i.e. anti-RNP, anti-ESmA, etc., but we have not looked into the relevance of this finding to false positivity of our Raji data.

2. Clinical Aspects

IC's are formed in vivo in normal subjects under certain circumstances (41, 129) and probably have useful normal immunologic function (141, 164, 194). In IC-mediated disease, due to imbalance in the normal regulatory immune functions, a heightened level of CIC is found especially in cases of persistent antigenemia, eg., chronic infections like SBE (13, 14), malaria (212), etc., with lymphomas (161, 194), melanomas (161), and autoimmune diseases like SLE (3, 5, 24, 25, 36, 66, 68, 103, 206) and rheumatoid arthritis (91, 95, 143). The pathogenicity of these high levels in CIC detected in the above situations is not understood and in those situations where there is poor correlations with disease activity such as melanoma (161), they may be epiphenomena and not pathogenic; in other situations such as SBE, they may cause the secondary manifestations of disease and CIC monitoring is also a valuable index of the effects of treatment.

We have here attempted to determine the value of CIC measurement in three selected, clinical situations and to understand better the relationship of CIC to immunopathology.

In hemodialysis patient sera, we have failed to confirm existence of anti-DNA antibodies either in free form or as DNA anti-DNA complex. This may dispel a misconception in the literature: that dialysis can cause DNA antibodies or immune complexes with DNA to develop. In cystic fibrosis, we have identified a subgroup of patients with poor pulmonary function and high CIC levels, and another similar group of severe illness with no CIC. The data bring out the necessity of further longitudinal studies and lead us to postulate that a group of cystic fibrosis patients developed IC-mediated lung injury. If this fact is borne out by further study in this laboratory and other laboratories, it provides a valid research question to be confirmed or refuted as to the necessary change in identification and treatment of this separate group's disease mechanism.

In the MS patients we have shown higher prevalence of CIC's in active states of disease and have shown, in our preliminary observations, that an unexpectedly high proportion of MS sera contains MBP as antigenic moiety in complexes eluted from Raji cells, using a radioimmunoassay inhibition test based on radiolabelled MBP and a known antiserum to MBP. These observations attest to the possible autoimmune nature of MS disease. One may speculate that:

i) CIC's could cause damage directly to MS plaques which, initially, in their evolution, are always perivenous in the CNS, and/or;

ii) CIC's could form as a secondary phenomenon with damaged MBP released in circulation as antigen and such complexes might influence other aspects of immunonegation, eg. by affecting antigen-specific suppressor cells. In this respect whether

the role of CIC is protective or detrimental has to be found out by longitudinal studies.

In this regard, we have some preliminary data showing that three patients with terminal MS did not have CIC by any methods whereas two patients just recovering from acute relapse had persistent CIC in a followup period close to one year. This is just the reverse pattern to that which seems to be emerging in CF where serum from all six terminal patients had raised levels of CIC;

iii) CIC could alter blood brain barrier to allow other mediators to be active in disease. Certainly further longitudinal studies are needed to determine the role of CIC in MS.

These three examples of the clinical application of CIC assays in disease show a trend, as well as the particular advantage of the Raji assay. In addition to being very sensitive, Raji cells have the potential for ag-ab isolation from serum (and perhaps from CSF). This may turn out to be their greatest value in future research, particularly when monoclonal antibodies become available as an additional refined tool with antigen specificity. To date there are no monoclonal reagents for the Pseudomonas strains, to MBP, to nDNA, to Streptococcal viridan strains (in SBE), but the prospects are exciting.

Summary and Conclusions

- The Raji assay for CIC has been developed and modified in regards to standardization procedures without heat-aggregated IgG. This gave reproducible results and minimum interassay variation.
- The modified Raji-RIA gave more positive results in different pathological sera in comparison to other CIC methods.
- The influence of higher level of serum IgG and antilymphocytic antibodies in causing false positive results in Raji RIA was examined and found to be minimal.
- In combination with other CIC techniques, Raji-RIA was used to detect immune complexes in three disease situations. In one of these, Raji cells were used for isolation and partial characterization of possible relevant antigen. Thus:
  - i) in hemodialysis, patients' sera were found not to contain DNA antibodies in free or complex form, clarifying a misconception in the literature;
  - ii) in cystic fibrosis, the incidence of CIC permits speculation that there may be a subgroup with immune complex-mediated lung injury, recognized by higher CIC levels;
  - iii) in MS, the incidence of CIC was significantly increased in active states of disease. Preliminary studies show a higher prevalence of MBP contains more complexes in MS than in controls. This attests to the autoimmune nature of the disease, proven by further longitudinal studies.

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APPENDIX 1

Diseases Associated with Immune Complexes

Autoimmune diseases
- Rheumatoid arthritis, Felty's syndrome, systemic lupus erythematosus, Sjögren's syndrome, mixed connective tissue disease, periarteritis nodosa, systemic sclerosis

Glomerulonephritis
- Exogenous and endogenous antigens

Neoplastic diseases
- Solid and lymphoid tumors

Infectious diseases
- Bacterial: Infective endocarditis, meningococcal infections, disseminated gonorrheal infection, recurrent infections in children, infected ventriculoarterial shunt, streptococcal infections, leprosy, syphilis
- Viral: Dengue hemorrhagic fever, cytomegalovirus infections, viral hepatitis, infectious mononucleosis, SSPE (subacute sclerosing panencephalitis)
- Parasitic: Malaria, trypanosomiasis, schistosomiasis, filariasis, toxoplasmosis

Other conditions
- Dermatitis herpetiformis and celiac disease, ulcerative colitis and Crohn's disease, myocardial infarcts, idiopathic interstitial pneumonia, cystic fibrosis, sarcoidosis, multiple sclerosis, amyotrophic lateral sclerosis, myasthenia gravis, uveitis, otitis media, atopic diseases, arthritis associated with intestinal bypass procedure for morbid obesity, sickle-cell anemia, thrombotic thrombocytopenic purpura, primary biliary cirrhosis, kidney and bone marrow transplantation, pregnancy, preeclamptic and eclamptic syndrome, Lyme arthritis, steroid-responsive nephrotic syndrome, xanthomatosis, vasectomy, oral ulceration and Behçet's syndrome, pemphigus and bullous pemphigoid, IgA deficiency, thyroid disorders, ankylosing spondylitis, iatrogenic diseases

From: Theofilopoulos, A.N., and Dixon, F.J. The biology and detection of immune complexes (Table VI, page 143). Adv. Immunol. 28: 89-220, 1979. (Ref. 194).

APPENDIX 2

	Quantity (ml)	Source	Catalogue No.
A. Raji cell culture medium:			
Eagles minimum essential medium (MEM)	800	GIBCO*	320-1090
Heated (56°C x 30 minutes) fetal bovine serum	100	GIBCO*	200-6140
L-glutamine (200 mM x 100)	10	GIBCO*	503
Non-essential amino acids (100 x)	10	Flow Lab**	16-810-49
Sodium pyruvate (100 mM)	10	Flow Lab**	16-820-49
Antibiotic-antimycotic solution	10	GIBCO*	600-5245
Penicillin 10,000 u/ml			
Fungizone 25 mcg/ml			
Streptomycin 10,000 mcg/ml			
5.6% sodium bicarbonate	25	Flow Lab**	16-882-49
TOTAL	1000		
B. Raji cell RIA wash medium:			
RPMI 1640 (with L-glutamine)	490	GIBCO*	310-1875
Antibiotic-antimycotic solution	5	GIBCO*	600-5245
Hepes Buffer (1 molar solution)	5	GIBCO*	380-5630
TOTAL	500		

* GIBCO - Grand Island Biologic Co., Grand Island, New York 14072, USA

** Flow Lab - Flow Laboratories Inc., 1710 Chapman Ave., Rockville, Maryland 20852, USA

Appendix 3:

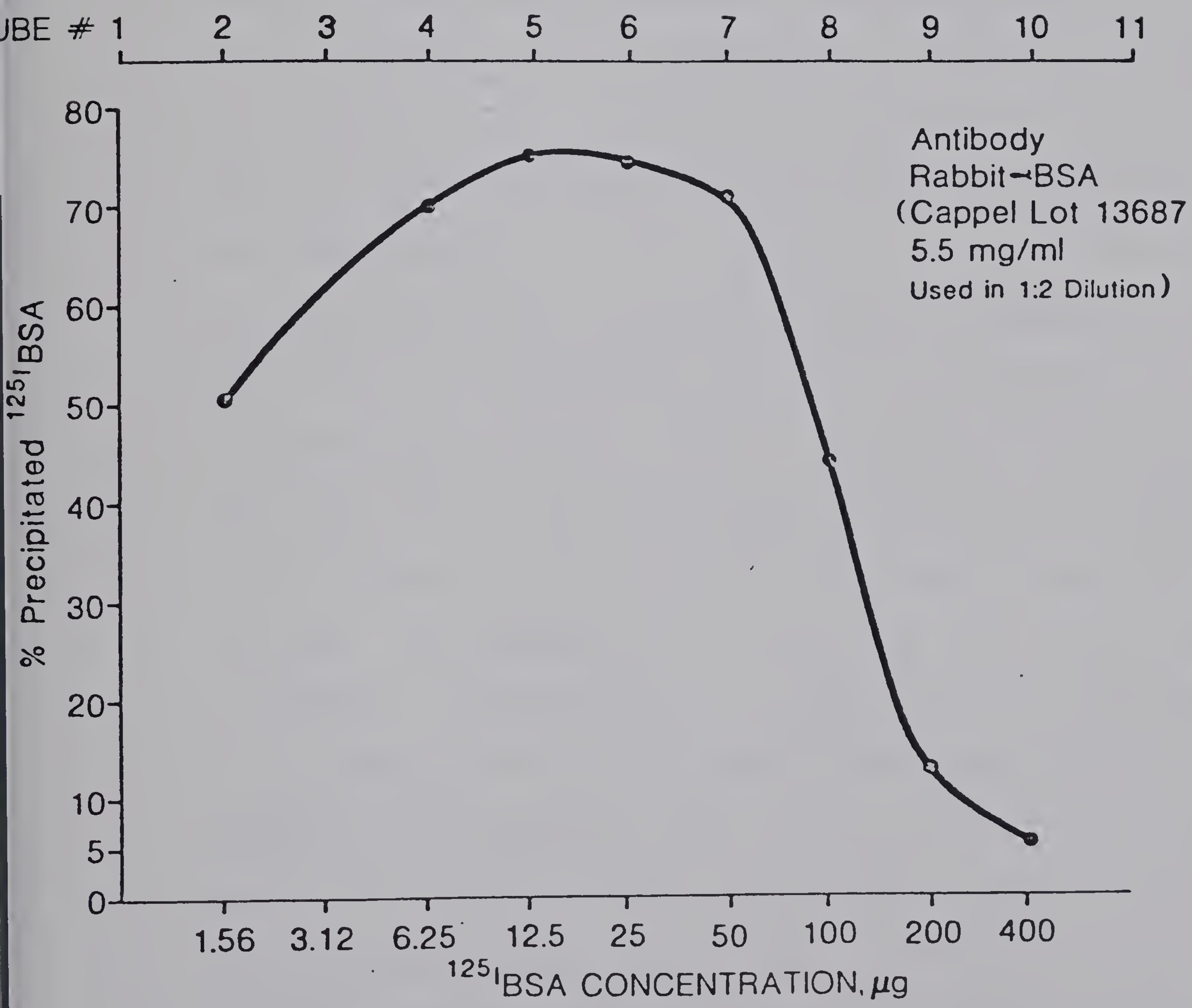
Preparation, isolation and characterization of *in vitro* complex (BSA- anti BSA) from Raji cell bound IC:

a) Preparation of BSA- anti BSA IC

A batch of Bovine serum albumin (BSA) [Cappel Lab., Lot #12149, Cat. #3002-0508] was radio labelled with ^{125}I by following the method of McConahey and Dixon (117). The concentration of the labelled BSA was 1.16 mg/ml with a specific activity of 75.1 μCi/μg. This was used as the antigen. Antibody was produced in rabbit custom made against this batch of BSA by Cappel Lab., Lot #13687, Cat. # 0202-0526, containing IgG fraction of antibody protein 5.5 mgm/ml. To a constant amount of this antibody diluted 1:2, an increasing amount of ^{125}I-BSA was added, in different tubes, and incubated at 37°CX 30 minutes followed by at 4°C overnight. At the end of the incubation period each tube was counted in a Gamma counter (total c.p.m.) and then centrifuged at 3000 rpm for 10 minutes at 4°C. Supernates were aspirated and precipitates from each tube were redissolved in 200 μl of PBS and transferred to marked fresh tubes. The resuspended precipitates were then dissolved in either 50% ammonium sulfate or 20% TCA (to avoid counts from free ^{125}I-BSA sticking to the walls of the tubes or counts from free ^{125}I) and recentrifuged as above. After centrifugation the supernates were discarded and precipitate counts from each tube were noted. Amount of ^{125}I-BSA present (precipitated) in each tube was expressed as the per cent of the total c.p.m. added in each tube (total count) and zone of equilibrium was determined. Representative experimental result is shown in Figure #22. This

Figure 22

BSA-BSA IN-VITRO COMPLEX



would show that for this batch of antisera at this combination ratio, the amount of BSA needed at equilibrium was 40 mgm. This was then used to calculate the amount needed to prepare *in vitro* ^{125}I BSA-anti BSA complexes in 5 times antigen excess. (For this batch it was taken as 200 mg.)

b) In Vitro IC uptake by the Raji cells

200 µl of above mentioned batch of ^{125}I- BSA and 200 µl of corresponding anti-BSA was added (200 µl, 1:2 dilution in PBS) and incubated at 37^{o}C for 30 minutes and at 4^{o}C for 1 hour. To 200 µl of this mixture was added 200 µl of normal human serum (NHS) diluted to 1:2 in PBS. (This was done to add NHS to the *in vitro* IC and also to use NHS as a source of complement, as Raji cells preferentially bind complement fixed complexes) and then incubated for 30 minutes at 37^{o}C. At the end of this incubation this 50 µl of the sample was added to 5X 10^{6} Raji cells and incubated for 45 minutes at 37^{o}C with intermittent shaking (same as Raji RIA for CIC). This sample was referred as BSA- anti BSA IC + C (C = complement). Another sample of *in vitro* sample was also prepared in the same way with the exception of adding heated NHS in the same volume etc. and labelled as BSA- anti BSA IC - C. Other controls consisted of adding neat ^{125}I-BSA, anti BSA, and NHS (1:4) to Raji cells separately and proceed as above. At the end of this incubation period Raji cells were washed X 3 times in wash media and pellet counts were noted. Representative experimental result is shown in Figure #7. This showed BSA- anti BSA IC + C bound

more effciently to the Raji cells than the same IC without complement (BSA- anti BSA-C), and poorer uptake by the ^{125}I BSA alone.

c) Acid elution of BSA - anti BSA complexes from Raji cells

This was done by following the method described by Theofilopoulos et al. 1978 (196). *In vitro* prepared ^{125}I BSA- anti BSA IC in 5 times antigen excess (as done in section b)) was added to 30 X 10^6 Raji cells in 100 µl of MEM medium without any Ca++ or protein added. Controls were set up similarly with ^{125}I = BSA, anti BSA, NHS, and BSA- anti BSA IC without any NHS and a tube containing Raji cells in medium only. Cells were then incubated at 37° for 45 minutes and then washed repeatedly in MEM medium without any protein added. After the last wash cells were resuspended in 200 µl of freshly prepared isotonic acid citrate buffer (pH 2.9 - 3.2) supplemented with rabbit IgG (1 mg/ml) and incubated at 37°C for 7 minutes. This was immediately followed by centrifugation at 3000 rpm at 4°C for 10 minutes and supernates were carefully collected (50 to 100 µl without disturbing the pellet. Eluates were then labelled and dialyzed for 18 hours against Tris buffer or PBS at pH 7.4. The dialyzed aliquots were then trated as samples for passing on to the polyacrylamide gels.

d) Sodium-dodecyl sulfate (Sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE)

This was done by following the procedure described by Lammelli (Nature, 227, 680, 1970). Initially it was performed on plate gels followed by auto radiography (done under the supervision of

Dr. D.L. Tyrrell). This took more than 7 days to complete one experiment. Later this was changed to column type of gels with no loss of sensitivity or specificity but additional advantage was that it took shorter time to complete the experiment (3 days) and direct counts were obtained by slicing the gels in a Gamma counter instead of auto radiography, which gave better quantitative results (auto radiogram vs. c.p.m.). Procedure for column gel was done under the supervision of Dr. T. Nihei, as described below:

i) 100 μl of eluted sample from Raji cells (step c)) was prepared for gel injection with 10 μl glycerol (66%). 10 μl merceptaethanol (10%), 10 μl SDS (10%) and heated to boiling for 3 minutes, and were applied to the polyacrylamide columns for run (4 - 5 hours). After the run gels were stained with 0.2% Coomassie Blue in 10% aceeic acid and destained with a mixture containing 40% methanol 10% aceteic acid and 50% water for overnight to 24 hours. After destaining the gels were sliced into equal pieces and counted directly in a Gamma counter. Representative result of elution of ^{125}I BSA from ^{125}I BSA anti BSA complexes is given in Figure # 8, Chapter III, Section 4.

SYSTEM OF CLINICAL EVALUATION OF THE PATIENTS WITH CYSTIC FIBROSIS

GRADING	UNITS	GENERAL ACTIVITY	PHYSICAL EXAMINATION	NUTRITION	X-RAY FINDINGS
Excellent (86-100)	25	Full normal activity; plays ball, goes to school regularly, etc.	Normal no cough, pulse and respirations normal; clear lung; good posture	Maintains weight and height at above 25th percentile; well-formed stools, almost normal; good muscle mass and tone	Clear lung fields
Good (71-85)	20	Lacks endurance and tires at end of day; good school attendance	Resting pulse and respirations normal; rare coughing or clearing of throat; no clubbing; clear lungs; minimal emphysema	Weight and height at approximately 15th to 20th percentile; stools slightly abnormal; fair muscle tone and mass	Minimal accentuation of bronchovascular markings early emphysema
Mild (56-70)	15	May rest voluntarily during the day; tires easily after exertion; fair school attendance	Occasional cough, perhaps in morning upon rising; respiration slightly elevated; mild emphysema; coarse breath sounds; rarely localized.	Weight and height above 3rd percentile; stools usually abnormal, large and poorly formed; very little if any abdominal distention; poor muscle tone with reduced muscle mass.	Mild emphysema with patcatelectasis; increase bronchovascular markings.
Moderate (41-55)	10	Home teacher; dyspneic after short walk; rests a great deal	Frequent cough, usually productive, chest retraction; moderate emphysema; may have chest deformity; rales usually present; clubbing 2 to 3+	Weight and height below 3rd percentile; poorly formed, bulky, fatty, offensive stools; flabby muscles and reduced mass; abdominal distention mild to moderate	Moderate emphysema widespread area of atelectasis with superimposed area of infection minimal bronchial ectasis.
Severe (40 or below)	5	Orthopneic, confined to bed or chair	Severe coughing spells; tachypnea with tachycardia and extensive pulmonary changes; may show signs of right-sided cardiac failure; clubbing 3 to 4+	Malnutrition marked: large protuberant abdomen; rectal prolapse; large, foul, frequent, fatty movements	Extensive changes with pulmonary obstructive phenomena and infection lobar atelectasis and bronchiectasis

APPENDIX 4: Shwachman Score J. Dis. Child. 96: 6, 1958.

APPENDIX 5

SDS Polyaerylamide Gel Electrophoresis of Raji Eluates and Radioummune Assay (RIA) for Myelin Basic Protein (MBP) (Established Procedure in the Laboratory of Dr. T.A. McPherson)(77)

I. Sample Handling for CIC Eluated From Raji Cells for, SDS Polyacrylamide Gel Electrophoresis

The preparation of samples and the electrophoretic procedure was as described in Section d of Appendix 3. 10 µl of sample were mixed with 50 µl 1% SDS and 40 µl of .01 m phosphate buffer. The mixture was boiled 90 sec. 5 µl of BME and 10 µl of glycerol, bromphenol blue were added. The sample was then underlayered on 10% polyacrylamide gels.

- gels were seen at 8 MAMPS per gel for four hours at room temperature.

- after the termination of the elctrophoresis, the gels were removed from the tubes, stained overnight in .1% Coomassie blue and destained by diffusion of excess stain into several changes of 10% acetic acid.

After destaining the gels were sliced into 3 mm slices. Each slice was macerated and protein eluted by incubation in 1.5 ml T_3 buffer for 18-24 hours at room temperature. Duplicate 0.5 ml aliquots of eluted protein were subjected to RIA.

II. RIA FOR MBP

A. <u>Production of Antibody</u>

Antibody to human BP was raised in female New Zealand white rabbits (77). Rabbits were bled from the marginal ear vein, the serum collected, stored at -20°C, and used without further purification.

B. Purification of Human Myelin Basic Protein

Human brains were obtained within 16-18 hours of death. BP was isolated using the large-scale extraction procedure.

C. Iodination of Antigen

BP was iodinated by the chloramine T procedure (77). Labelled BP was prepared every four weeks. The specific activity of the radio-labelled BP was about 70 μCi/μg.

D. Radioimmunoassay

Eluated samples from each gel slices were incubated with 0.5 ml of normal rabbit serum, a 0.2 ml concentrated T_3 buffer (0.2 M Tris, 1% Triton X100, 0.1% Trasylol, pH 7.2) and antibody (BP) (final dilution of 1:2,000. Incubation was carried out for 18 hours at 4°C. ^{125}I-BP was then added (0.2 ng - 25,000 cpm) and incubation continued for one hour at room temperature. The incubation was terminated by the addition of 1 ml of 8% silica gel as described by Hsiung and McPherson (77). The results were expressed as the percentage bound to antibody or as the percentage inhibition (percentage bound in the sample minus the percentage bound in the control).

E. Sensitivity and specificity of RIA

The silica gel RIA is capable of detecting a minimum of 2 ng of MBP/ml of CSF which is comparable to other assays in use. Specificity has been checked by including variable quantities of lysozomes or his-tones in the assay mixture and no cross-reaction was revealed. We have three categories corresponding to the amount of BP detected. A level less than 4 ng/ml is considered normal, where as a level greater than or equal to 4 ng BP/ml but less than 8 ng BP/ml is regarded as mildly elevated, and a level of 8 ng/ml or greater is considered definitely

elevated. Quantity found from Raji eluates were not always as high as could be found in CSF control run with labelled BP in isotonic acid buffer of Raji elution did not show any alteration in the sensitivity of MBP detection by the assay.